

**Applications of Enzymes to the Preparation of
Optically Active Compounds.**

By

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CONTENTS

List of figures.	vi
List of tables.	x
Acknowledgements.	xii
Declaration.	xiii
Summary.	xiv
Abbreviations	xv
Chapter 1: Aspects of Selectivity in Lipase-Catalysed	
Biotransformations.	1
1.1 Introduction.	1
1.1.1 Distribution of lipases.	2
1.1.2 Assay of lipase activity.	3
1.1.3 Mechanism of lipase action.	4
1.1.4 The use of biotransformations in organic	
synthesis.	7
1.1.5 Lipases and stereoselectivity.	8
1.1.6 Types of selectivity in lipase-catalysed	
reactions.	13
1.1.7 Aims of this review.	15
1.2 Pig Pancreatic Lipase.	16
1.2.1 PPL-catalysed reactions of primary alcohols.	17
1.2.2 Reactions of secondary alcohols using PPL.	25
1.2.3 Regioselectivity in PPL-catalysed reactions.	31
1.2.4 The use of PPL for resolution of chiral acids.	34
1.2.5 Reactions of lactones.	37
1.2.6 Conclusions on the use of PPL in organic	
synthesis.	39
1.3 The Lipase from <i>Candida cylindracea</i> .	43
1.3.1 Resolutions of secondary alcohols.	44

1.3.2 Esterolytic reactions with chiral acids.	54
1.3.3 Resolution of primary alcohols.	60
1.3.4 Regioselective reactions of carbohydrate substrates.	62
1.3.5 Concluding comments.	64
1.4 Lipase P from <i>Pseudomonas fluorescens</i> .	65
1.4.1 Lipase P-catalysed esterolytic reactions of secondary alcohols.	65
1.4.2 Esterolytic reactions of lipase P on primary alcohols.	74
1.4.3 Resolution of chiral acids.	76
1.4.4 Conclusions.	77
1.5 Other Microbial Lipases.	79
1.5.1 Lipase SAM II from <i>Pseudomonas sp.</i>	79
1.5.2 The lipase from <i>Mucor miehei</i> .	82
1.5.3 The lipase from <i>Aspergillus niger</i> .	86
1.5.4 the lipase from <i>Pseudomonas fragi</i> .	88
1.5.5 The lipase from <i>Pseudomonas aeruginosa</i> .	90
1.5.6. Wheatgerm lipase.	91
1.5.7 The lipase from <i>Chromobacterium viscosum</i> .	92
1.6 Typical Experimental Procedures For Biotransformations.	96
1.6.1 Hydrolysis with PPL.	96
1.6.2 Hydrolysis with PPL.	96
1.6.3 Hydrolysis with CCL.	97
1.6.4 Esterification with PPL.	98
1.6.5 Esterification with CCL.	98
1.6.6 General procedure for the lipase-catalysed transesterification of alcohols with enol esters.	99

1.7 Concluding Comments.	101
Chapter 2: Resolution of a Beta-Blocker Precursor.	109
2.1 Introduction.	109
2.2 Synthesis of the Racemic α -Chlorohydrin.	111
2.3 A Method of Chiral Analysis.	113
2.4 Enzymatic Hydrolyses of diester (119).	114
2.5 Transesterification of the α -Chlorohydrin.	118
2.6 Summary and Conclusions.	119
Chapter 3: Resolution of Methyl	
4-(<i>p</i> -chlorophenylthio)butanoate.	120
3.1 Introduction.	120
3.2 Synthesis of the Racemic Esters.	121
3.3 Hydrolysis of Methyl 3-acetoxy-4- (<i>p</i> -chlorophenylthio)butanoate.	122
3.4 Hydrolysis of Methyl 3-butanoyloxy-4- (<i>p</i> -chlorophenylthio)butanoate.	124
3.5 Transesterification Reactions.	130
3.6 Conclusions.	131
Chapter 4: Resolution of 3-Methyl-4-oxo-4- (4-aminobenzyl)butanoic acid.	132
4.1 Introduction.	132
4.2 Hydrolysis with PLE.	138
4.3 Interesterification with PLE.	142
4.4 Hydrolysis of the Corresponding Amide Ester with PLE.	143
4.5 Hydrolysis of the Methyl Ester with Lipases.	144

4.6	Interesterification with Lipases.	145
4.7	Reduction with Bakers' Yeast.	145
4.8	Development of a Reliable Method of Chiral Analysis.	147
4.9	Reduction with <i>Candida guilliermondii</i> .	147
4.10	Reduction with other Microorganisms.	148
4.11	Investigation of the Lability of the Product under the Fermentation Conditions.	150
4.12	Summary and Conclusions.	151
Chapter 5: Resolution of Ketones via Hydrolysis of a Corresponding Oxime Ester.		153
5.1	Introduction.	153
5.2	Preparation and Hydrolysis of Acetone Oxime Acetate.	154
5.3	Preparation and Hydrolysis of 2-Methyl cyclohexanone Oxime Acetate.	155
5.4	Preparation of 2,6-Dimethylcyclohexanone Oxime Acetate.	160
5.5	Enzymatic Hydrolysis of the Oxime Acetate.	170
5.6	Preparation and Enzymatic Hydrolysis of Norcamphor Oxime Acetate.	172
5.7	Preparation and Hydrolysis of Norcamphor Oxime Butanoate and Hexanoate.	179
5.8	Conclusions.	180
Chapter 6: Resolution of Chiral Ketones by Enzymatic Hydrolysis of an Enol Acetate.		182
6.1	Introduction.	182

6.2	Resolution of 2-Methylcyclohexanone.	184
6.2.1	Experiments with the Non-chiral Enol Acetate.	184
6.2.2	Experiments with the Chiral Enol Acetate.	186
6.3	Resolution of 2,6-Dimethylcyclohexanone.	189
6.4	Resolution of Norcamphor.	192
6.4.1	Synthesis of the Enol Acetate.	192
6.4.2	Enzyme-Catalysed Resolutions.	194
6.5	Summary and Conclusions.	197
Chapter 7:	Experimental Details.	199
7.1	Introduction.	199
7.1	Experimental to Chapter 2.	200
7.3	Experimental to Chapter 3.	208
7.4	Experimental to Chapter 4.	216
7.5	Experimental to Chapter 5.	223
7.6	Experimental to Chapter 6.	235
Chapter 8:	Bibliography.	243

LIST OF FIGURES.

Fig. 1	Variation of ee for different E values. A: ee of the remaining substrate, B: ee of the product.	10
Fig. 2	Computer-generated curves from equations 9 and 10 showing percentage enantiomeric excess for (A) product, and (B) remaining substrate as a function of percentage conversion at different values of E and K.	13
Fig. 3	Some primary alcohols resolved via PPL-catalysed reactions.	18
Fig. 4	Compounds hydrolysed nonselectively by PPL.	20
Fig. 5	Further primary alcohols resolved using PPL.	20
Fig. 6	Primary alcohols resolved using PPL.	21
Fig. 7	Alcohols resolved using PPL and lipase P.	22
Fig. 8	Secondary alcohols resolved using PPL-catalysed reactions.	25
Fig. 9	Hydrolysis of esters of bicyclic alcohols with PPL.	30
Fig. 10	Products of PPL-catalysed hydrolyses of fully esterified sugars.	31
Fig. 11	Hexosides acylated by PPL.	33
Fig. 12	Chiral acids resolved by PPL-mediated reactions.	34
Fig. 13	Esters of diacids hydrolysed by PPL.	36
Fig. 14	<i>Cis</i> and <i>trans</i> dioxolane compounds used as substrates for enzyme-catalysed hydrolyses.	36
Fig. 15	PPL-catalysed formation and hydrolysis of lactones.	37
Fig. 16	Substrates used in determination of the active site model of PPL.	40
Fig. 17	Active site model of PPL.	42

Fig. 18	Cyclic secondary alcohols resolved via CCL-catalysed reactions.	44
Fig. 19	Compounds with a norbornane type structure hydrolysed with high selectivity.	48
Fig. 20	Elucidation of the bridgehead requirements for a selective reaction.	49
Fig. 21	General substrate model for the hydrolysis of norbornane-type esters by CCL.	49
Fig. 22	Secondary alcohols resolved by CCL.	53
Fig. 23	A survey of CCL-catalysed reactions of acids.	55
Fig. 24	Primary alcohols resolved via CCL-catalysed reactions.	60
Fig. 25	CCL-catalysed reactions on sugars.	62
Fig. 26	Compounds deprotected using PPL and CCL.	63
Fig. 27	Secondary alcohols resolved with lipase P.	65
Fig. 28	Products of esterification reactions of secondary alcohols using lipase P.	69
Fig. 29	Cyclic secondary alcohols resolved with lipase P.	71
Fig. 30	Products of lipaseP-catalysed hydrolysis of cyclopentane diesters.	71
Fig. 31	Bicyclic esters hydrolysed by lipase P.	72
Fig. 32	Active site model for lipase P.	73
Fig. 33	Exo-substrates hydrolysed with high selectivity using lipase P.	73
Fig. 34	Newman projections of <i>endo</i> and <i>exo</i> substrates.	74
Fig. 35	Primary alcohols resolved by lipase P-catalysed reactions.	75
Fig. 36	Chiral acids resolved with lipase P.	76
Fig. 37	Compounds resolved using lipase SAM II.	79
Fig. 38	Cyanohydrins resolved with lipase SAM II.	81

Fig. 39	Relative rates of esterification of octanol with various acids.(a) study of the effect of branching in the acid chain.(b) comparison of the rates of reaction of cyclohexyl and phenyl-substituted acids.	83
Fig. 40	Products of MML-catalysed ester hydrolysis.	85
Fig. 41	Products of hydrolytic reactions catalysed by the lipase from <i>A. niger</i> .	87
Fig. 42	Products of reactions catalysed by lipase from <i>P. aeruginosa</i>	91
Fig. 43	Regioselective reactions catalysed by lipase from <i>C. viscosum</i>	93
Fig. 44	Minimal steroid structure.	94
Fig. 45	Alcohols resolved by PPL-catalysed hydrolysis of an ester derivative.	102
Fig. 46	Esters resolved by lipase P.	104
Fig. 47	Generalised substrate esters for hydrolysis.	107
Fig. 48	Structures of early beta-blockers.	109
Fig. 49	General beta-blocker synthesis.	110
Fig. 50	The beta-blocker precursor to be resolved.	111
Fig. 51	Mosher's ester of the α -chlorohydrin.	114
Fig. 52	Products recovered from the enzymatic hydrolysis.	115
Fig. 53	Autotitrator traces for the hydrolysis with and without surfactant.	126
Fig. 54	NMR spectra for the optically active alcohol (a) without shift reagent, (b) with shift reagent, (c) with shift reagent and added racemic alcohol.	128
Fig. 55	Examples of PLE-catalysed reactions.	133
Fig. 56	The compound to be resolved.	134
Fig. 57	The vasodilator (122) and the target drug molecule (123).	134
Fig. 58	General formula of unsaturated compounds reduced	

	by bakers' yeast.	135
Fig. 59	Compounds obtained by reduction of a double bond using bakers' yeast.	136
Fig. 60	Examples of optically pure compounds obtained by reduction with <i>C. kluyveri</i> .	137
Fig. 61	Conformation of 2-methylcyclohexanone oxime.	156
Fig. 62	Minimised conformations (PC MODEL) for 2,6-dimethylcyclohexanone oxime: (a) <i>cis</i> diaxial, (b) <i>cis</i> diequatorial (c) <i>trans</i> .	163
Fig. 63	Part of the ^1H NMR spectrum of 2,6-dimethylcyclohexanone decoupled at C-2 and C-6 in turn.	166
Fig. 64	The enantiomers of 2,6-dimethylcyclohexanone oxime.	168
Fig. 65	The two conformational isomers of norcamphor oxime.	173

LIST OF TABLES.

Table 1	PPL-catalysed esterification of secondary alcohols.	27
Table 2	Influence of acid and alcohol in resolution.	34
Table 3	Influence of the acid moiety in resolution of a secondary alcohol.	54
Table 4	Study of the effect of acylating agent on the enantioselectivity of CCL-catalysed esterifications.	61
Table 5	Investigation of the effect of variation of the acid component in the hydrolysis of esters of a chiral alcohol.	67
Table 6	Esterification of various secondary alcohols catalysed by lipase P.	70
Table 7	Transesterification of esters of secondary alcohols catalysed by lipase P.	70
Table 8	Number of reactions on each substrate type catalysed by each of the common enzymes.	106
Table 9	Lipases used for a screening experiment for hydrolysis of ester (126).	144
Table 10	Results of the reductions performed with bacteria and bakers' yeast.	149
Table 11	Comparison of predicted ^{13}C NMR chemical shifts (δ_{pred}) for major and minor isomers of 2-methylcyclohexanone oxime with those observed (δ_{obs}).	157
Table 12	Enzymes used in screening for hydrolysis of 2-methylcyclohexanone oxime acetate.	159
Table 13	Predicted values for the ^{13}C NMR shifts of the <i>cis</i> and <i>trans</i> isomers.	162
Table 14	Predicted and experimental ^{13}C NMR shifts for the ring carbon atoms of 2,6-dimethylcyclohexanone oxime (major isomer).	162

Table 15	Simulated ^1H NMR data for the two <i>cis</i> conformations.	167
Table 16	Frequencies and relative intensities of the observed peaks.	168
Table 17	Enzymes used in the screening for hydrolysis of the oxime acetate.	170
Table 18	Correlation of the data reported by Hawkes with experimentally determined data for the major oxime isomer.	174
Table 19	Assignment of the ^{13}C NMR signals of norcamphor.	174
Table 20	Results of the screening experiment for hydrolysis of the enol acetate.	188
Table 21	Lipases used in the screening experiment for enantioselective hydrolysis of the enol acetate of 2,6-dimethylcyclohexanone.	191
Table 22	Lipases used for the transesterification reaction	192
Table 23	Results of a screening experiment for the transesterification of norcamphor enol acetate.	196
Table 24	Results of further screening work on the transesterification of norcamphor enol acetate.	196

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DECLARATION

The work described in this thesis is the original of the author, except where acknowledgement has been made to results and ideas previously published. It was carried out in the Department of Chemistry, University of Warwick between October 1986 and September 1990 and has not been submitted previously for a degree at any institution.

SUMMARY.

The introduction to this thesis is in the form of a review entitled 'Aspects of Selectivity in Lipase Catalysed Biotransformations'. Each of the most widely used lipases have been discussed. The reactions of each lipase have been extensively reviewed with the aim of establishing whether any trends have appeared in the characteristics of compounds accepted as substrates.

The remaining chapters cover five unrelated studies in areas of resolutions of chiral compounds using biotransformations.

In Chapter 2 the resolution of a β -blocker precursor was attempted via lipase-catalysed hydrolysis. 1-Chloro-2-hydroxy-3[4(2-acetoxyethyl)phenoxy]propane was obtained in high enantiomeric excess from hydrolysis of the corresponding butyrate ester with lipases from *Mucor* or *Rhizopus* sp. Yields were low however, owing to enzyme inhibition by the butyric acid byproduct.

In Chapter 3 the resolution of methyl 3-hydroxy-4-(p-chlorophenylthio)-butanoate was carried out. Hydrolysis of the corresponding butanoate ester with lipase P gave the *R* enantiomer of the desired compound in high enantiomeric excess. Transesterification of the racemic alcohol with vinyl acetate, again catalysed by lipase P, furnished the opposite enantiomer in 62% ee.

Chapter 4 is concerned with the resolution of a chiral acid, namely 3-methyl-4-oxo-4(4-aminobenzyl)butanoic acid. This was attempted by hydrolysis of an ester using pig liver esterase and various lipases and via microbial reduction of the corresponding unsaturated compound. The reactions were all found to be non-stereoselective.

Chapters 5 and 6 discuss novel methods for the enzymatic resolution of ketones.

The enantioselective enzymatic hydrolysis of oxime esters is discussed in Chapter 5. The resulting optically enriched oximes may readily be cleaved to the ketones. This method was unsuccessful in the resolution of the 2-methyl- and 2,6-dimethylcyclohexanones. A low enantiomeric excess was achieved in the resolution of norcamphor, and attempts to improve this using a purified enzyme and by variation of the ester chain were unsuccessful. However, this represents the first example of the indirect enzymatic resolution of ketones.

In Chapter 6 the enantioselective hydrolysis of enol acetates of three ketones was attempted. This method of resolution was unsuccessful in the resolution of 2-methyl and 2,6-dimethylcyclohexanones. In the case of norcamphor the ketone was obtained in low enantiomeric excess.

ABBREVIATIONS.

¹H NMR Proton Nuclear Magnetic Resonance Spectroscopy.

¹³C NMR Carbon Nuclear Magnetic Resonance Spectroscopy.

TLC Thin Layer Chromatography.

HPLC High Pressure Liquid Chromatography.

GLC Gas Liquid Chromatography.

IR Infra red spectroscopy.

s singlet.

d doublet.

t triplet.

q quartet.

m multiplet.

br broad.

J coupling constant (Hz).

ppm parts per million.

TMS tetramethylsilane.

ee enantiomeric excess

α measured rotation.

[α]_D specific rotation measured at 589 nm.

c concentration (g/100 ml).

DMSO Dimethyl sulphoxide.

THF Tetrahydrofuran.

TMEDA Tetramethylethylenediamine.

DMAP Dimethylaminopyridine.

CHAPTER 1

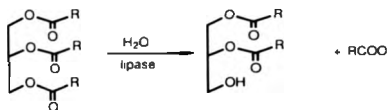
ASPECTS OF SELECTIVITY IN LIPASE-CATALYSED

BIOTRANSFORMATIONS.

1.1 INTRODUCTION

Lipases (E.C.3.1.1.3.) are a member of the family of carboxylesterase hydrolytic enzymes and in nature catalyse the hydrolysis of fatty acid esters of glycerol according to the general equation below:

Scheme 1. Hydrolysis of triglycerides.



Some lipases do catalyse further hydrolysis of the diglyceride to monoglyceride or even glycerol, and catalysis may occur at a pH other than that which generates the fatty acid in its ionised form. Other esters are also susceptible to attack by lipases. Lipases may be distinguished from other carboxylesterases by the physical nature of the substrate required for catalysis. Because of the fatty nature of their true substrates, lipases will act upon an emulsion of a substrate, and display very low activity when the substrate is fully dispersed in water.¹ For example pure pancreatic lipase is unable to catalyse the hydrolysis of triacetin at a low concentration but a high activity develops when the concentration is increased such that emulsified particles begin to form.²

The lipase from *Candida cylindracea* hydrolysed octyl 2-chloropropionate but not the methyl ester of the same acid. This was attributed to the fact that the methyl ester was completely dissolved while the octyl ester existed as an emulsion.²

1.1.1 Distribution of lipases.

Lipases have been found in and isolated from animal, plant and microbial sources, and lipases from each class have been used in biotransformations.

Animal lipases.

Three types of lipase have been defined in animals: the lipases discharged into the digestive tract, tissue lipases and milk lipases.¹ Among digestive lipases, that of the pancreas has been isolated and used for biotransformations. Despite its low abundance compared to other pancreatic enzymes (2.5% of the total pancreatic protein in the pig¹) this enzyme is important in the digestion of fats. Pig pancreas contains a high activity of lipase compared to that from other animals (13,000 U/g compared to 4,300 U/g in the horse pancreas, 2,800 U/g in the cow and 1,700 U/g in sheep).⁴ Pig pancreatic lipase has been purified and utilised extensively in biotransformations.

Plant lipases

Few studies have so far been made on plant lipases but high lipolytic activity has been demonstrated in germinating seeds.⁵

The lipase isolated from wheatgerm has been utilised in some biotransformations. A lipase has also been isolated from oats.

Microbial lipases.

Lipases are found widely in bacteria, yeasts and fungi. Most of these enzymes are exocellular (i.e. excreted into the culture medium) and conditions may be optimised for lipase production.^{5a} In some cases, for example with *Candida paratipolytica* and *Candida cylindracea* the biosynthesis of lipase can be induced by addition of glycerides, cholesterol or surfactants to the culture medium. In other cases, addition of such inducers inhibits biosynthesis of the lipases (eg with *Pseudomonas fragi* and *Geotrichum candidum*).¹ Many microbial lipases are now commercially available as powders which can simply be used as a chemical reagent thus widening their appeal to the synthetic organic chemist.

1.1.2 Assay of lipase activity

The lipase-catalysed hydrolytic reaction may be followed either through disappearance of ester or formation of alcohol or acid. Various insoluble substrates have been utilised in lipase assays, including Tween 80, *p*-nitrophenylacetate and *p*-nitrophenylbutyrate. The water soluble Tween 20 has also been used.⁶

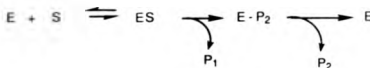
Methods of choice for routine work include titration of fatty acids either by conventional methods or by use of a recording pH-Stat and the continuous titration of acids released from an olive oil emulsion stabilised by gum arabic. Emulsions of tributyrin have also been used with satisfactory results. More

recently high speed stirring of a two-phase system (isooctane/aqueous buffer) has been proposed. This appears to be useful for assay of solid lipid substrates as well as liquids such as olive oil.^{6a}

1.1.3 Mechanism of lipase action.

The mechanism of lipase hydrolysis has been considered to be the same as for serine proteases such as α -chymotrypsin which hydrolyses peptide bonds via an acyl enzyme intermediate.

Scheme 2. Enzyme mechanism involving an acyl-enzyme intermediate.



where P_1 is the liberated alcohol, P_2 is the product and $E-P_2$ is the acyl enzyme intermediate.

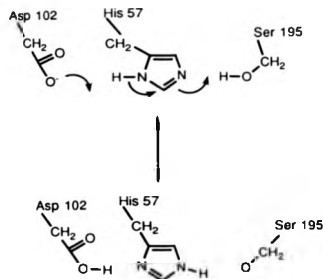
Inactivation of α -chymotrypsin with diisopropylphosphofluoridate (specific for serine) implicated a serine (residue 195) in the mechanism. Affinity labelling studies further showed that Histidine 57 was involved. X-Ray studies showed that His 57 and Serine 195 were adjacent and that the carboxyl side chain of Aspartate 102 was also close by.^{7a,b} Further X-ray and chemical studies elucidated the "charge relay network" of the catalysis. The catalytic triad has also been demonstrated by NMR experiments with α -lytic protease (another serine protease) in organic solvent.⁸ His 57 acts as a general base catalyst, accepting a proton from Ser 195.

rendering that residue more nucleophilic and allowing attack on the carbonyl group of the peptide to be cleaved.

A transient tetrahedral intermediate forms as Asp 102 is precisely oriented to partially neutralise the charge on the imidazole ring. The stored proton is donated to the N-component of the peptide bond to form the acyl enzyme intermediate.

In the deacylation step the charge-relay system draws a proton away from a water molecule, generating a hydroxyl ion which attacks the acyl enzyme intermediate in the reverse of the acylation step. The charge relay system is shown below.⁹

Scheme 3. The charge relay system of α -chymotrypsin



There is now much evidence to indicate that lipases operate by a similar mechanism. Early claims that pancreatic lipase was a "sulphydryl enzyme" were discredited, although the enzyme was shown to have two sulphur groups; one near the site

responsible for attracting the enzyme to the hydrophobic interface and the other near the active site.¹⁰

It was an early suggestion that a serine or threonine residue might be the acylation site,¹¹ and later studies of the inactivation of pig pancreatic lipase with bile salts proved that a serine residue was indeed involved.¹² Histidine has also been implicated in the mechanism of pig pancreatic lipase.^{13,14} Kinetic studies on lipase reactions, and trapping and isolation of the acyl-enzyme intermediate also support this acylation and deacylation mechanism.^{15,16}

Recently X-ray crystallographic studies of human pancreatic lipase and the lipase from *Mucor miehei* have shown that both of these enzymes have a catalytic triad similar to that of α -chymotrypsin.^{17,18} Human pancreatic lipase has an Asp-His-Ser triad chemically analogous to that of the serine proteases. Serine 152 is the active serine (as is the case for the porcine enzyme) and the triad is covered by a surface loop. The same situation has been revealed for the fungal lipase from *Mucor miehei*, with Ser 144, His 257 and Asp 203 forming the triad which is again buried under a loop folded over the surface. A central β -pleated sheet in the structure also bears a close resemblance to the structure of carboxypeptidase A, another serine protease.

This evidence confirms that the lipases act *via* a mechanism similar to that described above for the serine proteases.

1.1.4 The use of biotransformations in organic synthesis.

Several reviews have appeared over recent years which describe some of the applications of biotransformations in organic synthesis. Lipase reactions are included in such literature.^{19,20,21}

A wide range of lipases is utilized in biotransformations. Amongst them pig pancreatic lipase has been most widely used. As the natural substrates of lipases are fatty acid esters of glycerol it follows that lipases are most frequently used to resolve esters of chiral alcohols by enantioselective hydrolysis. However examples are also known of highly selective hydrolyses of esters of chiral acids. Hydrolytic reactions may be performed in a buffer system or it may be necessary to add a cosolvent or to use a biphasic system to overcome low solubility of the substrate. Another technique used to overcome solubility problems is emulsification with polyvinyl alcohol, although the effects of the surfactant may not always be beneficial as it may compete for the interfacial region and cause inhibition.²²

Lipases are also stable and active in a wide range of organic solvents and have been used for resolution procedures *via* esterification and transesterification reactions in organic media. Several reviews and articles have appeared in which enzyme applications in organic media have been discussed.²³⁻²⁹ The main problems associated with reactions in organic solvents is that they are much slower than reactions in aqueous media and that the reactions are reversible. Various approaches have been used to circumvent these problems.

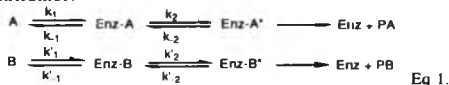
A number of methods have been devised to enhance selectivity in lipase-catalysed reactions and these have been reviewed recently.³⁰

1.1.5 Lipases and stereoselectivity.

The enantioselectivity of lipase-catalysed resolution is due to competition between the two enantiomers of the substrate. In an ideal case of enantiodiscrimination the (*R*) or (*S*) enantiomer of the substrate will be accepted by the enzyme while the other enantiomer binds very weakly to the enzyme or not at all. In this instance both isomers can be obtained essentially enantiomerically pure. In some cases one isomer may be bound very tightly and irreversibly to the enzyme, acting as a competitive inhibitor. In this instance the other isomer is selectively but incompletely converted to the product as more and more of the enzyme is bound in the unproductive complex. Generally however, both enantiomers are converted to product but one reacts very much faster than the other. In such cases in order to compare the selectivities of different enzymes and substrates the enantiomer ratio *E* has been introduced. This is a biochemical constant for the enzyme-catalysed reaction on a particular substrate that is independent of time and substrate concentration.³¹

In hydrolysis the reaction is essentially irreversible. The substrate enantiomers act as competitive inhibitors of each other. However, the concentration of the faster reacting enantiomer (designated A) decreases more rapidly than that of B, the slower reacting enantiomer. This releases B from the effects of inhibition by A with a subsequent increase in

hydrolysis of B. Equation 1 shows the reactions of each enantiomer.



(PA and PB are products from enantiomers A and B.)

When the enzyme system obeys Michaelis Menten kinetics

($k_{-1} \gg k_2$ $k'_{-1} \gg k'_2$) the enantiomer ratio or E value is

determined from the relative rate constants of binding and catalysis.

$$E = \frac{\ln\left(\frac{A}{A_0}\right)}{\ln\left(\frac{B}{B_0}\right)} = \frac{\left(\frac{k_{cat}}{K_m}\right)A}{\left(\frac{k'_{cat}}{K_m}\right)B} \quad \text{Eq 2}$$

A_0 and B_0 are the concentrations of A and B at zero time. It is possible to calculate the ee of the products PA and PB in terms of the "conversion ratio"

$$c = 1 - \frac{(A+B)}{(A_0+B_0)} \quad \text{Eq. 3}$$

This gives the expression for the enantiomer ratio given below:

$$E = \frac{\ln\left(\frac{[1-c][1-ee_s]}{[1-c][1+ee_s]}\right)}{\ln\left(\frac{[1-c][1-ee_s]}{[1-c][1+ee_s]}\right)} \quad \text{Eq. 4}$$

where c =conversion

ee_s = ee of the remaining substrate.

The conversion may be taken from GLC or HPLC analysis of the reaction mixture, or more accurately from the relationship:

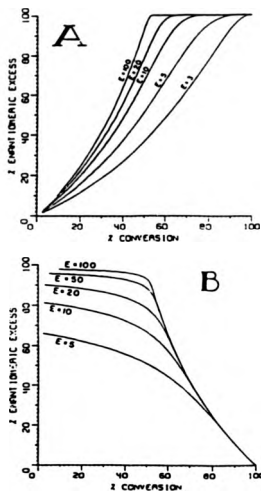
$$c = \frac{ee_s}{ee_s + ee_p} \quad \text{Eq. 5}$$

where ee_p = ee of the product.

This equation can be plotted graphically and Fig 1 shows plots of percentage enantiomeric excess versus the percent conversion for various enantiomer ratios, E. A shows the ee of the remaining substrate and B shows the ee of the product.

Figure 1 . Variation of ee for different E values.

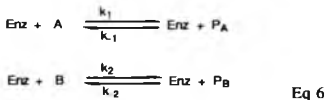
A : ee of the remaining substrate, B : ee of product.



From Fig. 1A it can be seen that the ee of the unhydrolysed substrate increases as the reaction proceeds, eventually reaching a value of 100%. However, the degree of conversion required to attain an ee of 100% is dependent on the enantiomer ratio E. At high values of E, an ee of 100% of the remaining substrate is attained at just over 50% conversion, but at low values of E, high optical purity of the remaining substrate is only attained at a much higher degree of conversion. In such cases, yield is sacrificed in order to attain high optical purity. With regard to product, the reverse situation holds. In all cases (Fig. 1B), the ee falls sharply after 50% conversion. At high E values, the product has high optical purity up to 50% conversion, whereas at low E values, the optical purity is defined at time zero and falls off rapidly, with a more rapid decrease, the lower the E value. The significance of the curves shown in Fig. 1 is that they allow one to predict the degree of conversion required in a system of known E value to achieve the best compromise between degree of conversion (yield) and optical purity of either substrate or product.

As an indication, an enzyme with an E value >100 would be considered to be highly selective, but effective resolutions can be carried out in systems where E has a value as low as 10. However, in such cases yield will be sacrificed in order to achieve high optical purity, as noted above.

This relationship does not apply in the case of biotransformations in low-water systems when the reactions are reversible. Equation 6 describes the esterification of the enantiomers of an alcohol in the presence of an excess of acyl donor or acceptor.



The action of the enzyme is to speed up the attainment of equilibrium; it does not change the position of equilibrium. The equilibrium constant therefore depends only on the initial and final states and the equilibrium constants for the enantiomers should be identical.

$$K = \frac{k_{-1}}{k_1} = \frac{k_{-2}}{k_2} = \frac{A}{P_\text{A}} = \frac{B}{P_\text{B}} \quad \text{Eq 7}$$

Thus equation 7 shows that the preferred chirality for forward and reverse reactions is the same. If A is the enantiomer which is esterified faster P_A would be the faster reacting enantiomer if the reaction were driven in reverse by hydrolysis or by transesterification against an achiral alcohol.

An expression incorporating the thermodynamic parameter K is required for calculation of the enantiomer ratio E.

$$E = \frac{\ln \left[1 - (1+K) \left(1 - \frac{A}{A_0} \right) \right]}{\ln \left[1 - (1+K) \left(1 - \frac{B}{B_0} \right) \right]} \quad \text{Eq 8}$$

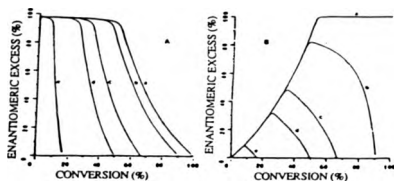
To correlate the conversion with the enantiomeric excesses of substrate and product the following equations have been derived.

$$E = \frac{\ln \left[1 - (1+K) (c + e e_s \{ 1 - c \}) \right]}{\ln \left[1 - (1+K) (c - e e_s \{ 1 - c \}) \right]} \quad \text{Eq 9}$$

$$E = \frac{\ln \left[1 - (1+K) c (1 + e e_p) \right]}{\ln \left[1 - (1+K) c (1 - e e_p) \right]} \quad \text{Eq 10}$$

The graphs shown in Fig 2 have been reproduced from the work of Sih et al³⁰ and show computer curves generated from equations 9 and 10. This provides an overview of the interrelationships between c , ee_s and ee_p for fixed values of E and K .

Figure 2. Computer generated curves from equations 9 and 10 showing percentage enantiomeric excess for (A) product and (B) remaining substrate as a function of the percentage conversion at different values of K for an E value of 100.



The values of K were (a) 0, (b) 0.1, (c) 0.5, (d) 1.0, (e) 5.0.

These plots show that a small increase in the value of K has a pronounced effect on the enantiomeric purity of product and remaining substrate even for a system with a high E value.

1.1.6 Types of Selectivity in Lipase-Catalysed Reactions.

Since lipases have mainly been used in the kinetic resolution of esters of chiral alcohols, a major consideration concerns selectivity with respect to primary and secondary alcohol sites. Tertiary alcohols appear not to act as substrates in lipase

catalysed esterolytic reactions. A major distinction therefore is between those lipases that catalyse esterolytic reactions of primary alcohols and those that catalyse esterolytic reactions of secondary alcohols. Such distinctions are rarely, if ever absolute. However, the literature contains clear cases when certain lipases have proved to be more effective than others in catalysing enantioselective reactions of primary alcohols, and another group has been found, in practice, to be more effective in catalysing enantioselective reactions of secondary alcohols. Since the normal substrates of lipases are (presumably) glycerides, i.e. glycerol esters of long-chain carboxylic acids it is not surprising that enantioselectivity in hydrolysis of esters of chiral alcohols often shows a dependence on chain length of the carboxylate component. Enantioselectivity can therefore be optimised by correct choice of acyl component.

A key factor in esterolytic transformations is the absolute sense of chiral discrimination displayed by the enzyme. As noted above, X-ray crystallographic structures of lipases have only recently been published and even in these cases the full atom coordinates have not been released. Accordingly, for mapping of the active site, one is still dependent mainly upon structure-activity studies. However, sufficient data have been accumulated for certain enzymes to permit the construction of outline models of the active sites.

Similar, but much less extensive data are available with respect to enantioselective reactions of esters of chiral acids.

These are the principal types of selectivity to which reference will be made in the following review.

1.1.7 Aims of this review.

Having briefly introduced the field of lipase catalysed biotransformations the aim of this review is to discuss the reactions and resolutions catalysed by various lipases. A survey of the extensive literature shows that lipases have widely different selectivities with respect to substrate. However, very few ground rules exist whereby one might predict the most appropriate lipase for use in a given application. The objective of this review is to draw together the diffuse information in the literature and to attempt to extract from it such indications as there may be of aspects of the selectivity of the various enzymes. The literature included was obtained from a search of the Chemical Abstracts Database, but it is not intended to be a fully comprehensive survey. For instance most of the patent literature has not been included and the search specifically excluded the reactions of oils and fats. Many reactions have been performed with more than one lipase and so may appear more than once in the review.

1.2 PIG PANCREATIC LIPASE

Pig pancreatic lipase is the only mammalian lipase in common use in biotransformations. It has already been mentioned that pancreatic lipase constitutes only a small proportion of the total pancreatic protein and this has led to considerable difficulty in its purification, mainly because of the close association of lipids with the enzyme. The most satisfactory techniques therefore start from defatted pancreas powder. Two molecular forms with lipase activity can be separated, but the isoelectric points, amino acid compositions and specific activities of the two forms differ little from each other.³² The composition of the rat enzyme is also very similar to that of the porcine enzyme.¹ The commonly used pig pancreatic lipases are crude enzyme preparations (for example "pancreatin") containing as little as 17% protein, not all of which is necessarily the lipase. Thus the biotransformation may be catalysed by another enzyme present in the mixture. Attempting to improve a biotransformation using a purified PPL may lead to the desired activity declining or disappearing altogether. In reports of PPL-catalysed hydrolysis of amino acid esters³³ and peptide synthesis³⁴ purified PPL did not catalyse the reactions at all and crude PPL exhibited a lower activity than papain. This, coupled with the low yields obtained, suggests that the reactions are, in fact, catalysed by the protease impurities in the crude PPL. Despite such limitations PPL has been utilised extensively for biotransformations. It has been demonstrated that PPL when dried becomes heat stable and may be used in organic solvents

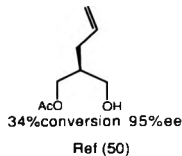
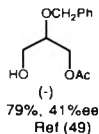
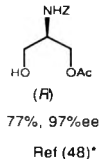
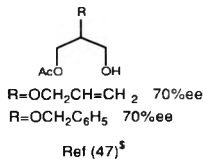
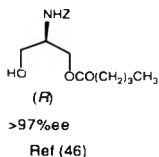
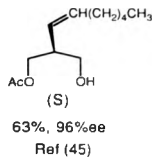
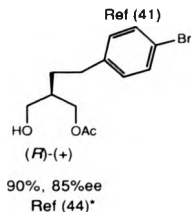
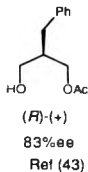
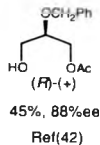
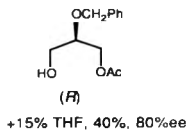
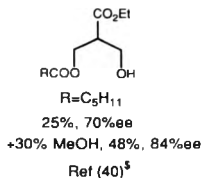
at temperatures of up to 100°C.³⁵ PPL has also been modified by alkylation to render it more active.³⁶ However, because crude PPL has been used in so many biotransformations, interpretation of the results is made difficult by the absence of precise information on the nature of the enzyme(s) actually catalysing the observed reaction.

It has been reported that pancreatin hydrolyses the esters from sn-1 and sn-3 positions of 3-O-octadecyl-1,2-dioctadecenoyl-sn-glycerol at equal rates,³⁷ showing that it is selective for esters of primary alcohols. Thus it is not surprising to find that PPL has been used extensively for reactions of primary alcohols. PPL has been utilized in the selective acylation of a primary alcohol function in the presence of a secondary alcohol moiety³⁸ and has also been shown to acylate an amino function in preference to a secondary alcohol.³⁹

1.2.1 PPL-catalysed reactions of primary alcohols.

In Fig. 3 are shown some primary alcohols resolved using PPL. In each case (except when specified), the structure shown corresponds to that of the enantiomer most rapidly released by hydrolysis of the corresponding ester.

Figure 3. Primary alcohols resolved via PPL-catalysed reactions.



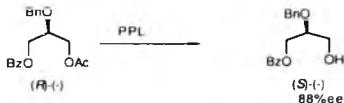
*=product of an esterification reaction, not hydrolysis.

§=Absolute configuration not determined.

It may readily be seen that variation in the substituent at the 2-position has a dramatic effect on the selectivity of the reaction.

In one example of a hydrolytic reaction,⁴⁵ variation of the solvent was found to increase the enantiomeric excess from 85%ee in water/THF 85:15, to 93%ee in water/ *tert*-butanol 90:10, to 96%ee in water/ diisopropyl ether 85:15. A diisopropyl ether : water solvent system has also been used elsewhere.⁴² In this example the product monoester was reesterified and rehydrolysed to give the opposite enantiomer of the corresponding benzyl ester (Scheme 4).

Scheme 4. Hydrolysis of the corresponding benzyl ester⁴²



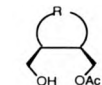
Hydrolysis of the following compounds resulted in racemic alcohols⁴³(Fig. 4).

Figure 4. Compounds hydrolysed non selectively by PPL.

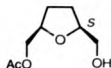


In Fig. 5 are shown more examples of primary alcohols which have been resolved using PPL-catalysed reactions.

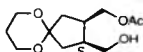
Figure 5. Further primary alcohols resolved using PPL.



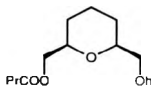
$R = (\text{CH}_2\text{CH}=\text{CH})_2$
(-)
96%, >99%ee
Ref (51)



41%ee
Ref (52)



87%ee
Ref (53)

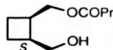


55%ee
Ref (54)

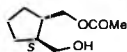


84%, 94%ee

Ref (55)

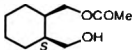


80%, 96%ee



90%, 89%ee

Ref (55)

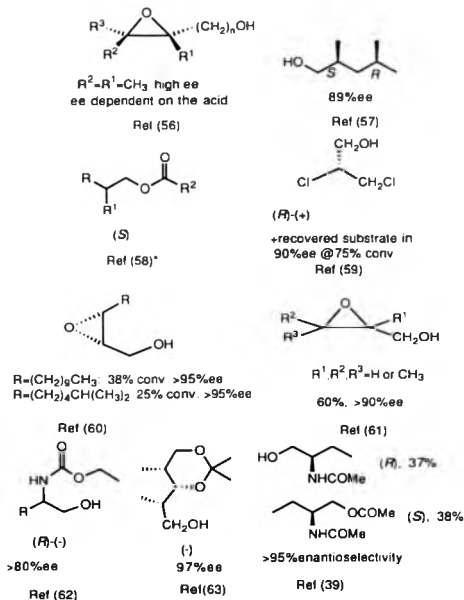


67%, 87%ee

The effect of ring size in this structure type has been studied.^{51,52} PPL hydrolysed all esters of cyclic compounds tested with almost complete selectivity.

Fig. 6 shows further primary alcohols resolved via PPL-catalysed reactions.

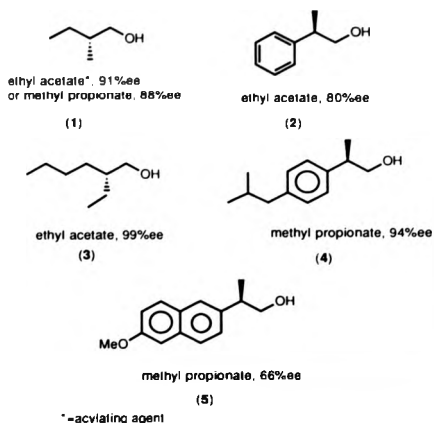
Figure 6. Primary alcohols resolved with PPL.



* = Product of esterification rather than of hydrolysis.

Bianchi et al⁵⁸ have described resolution of primary alcohols via transesterification with ethyl acetate, methyl propionate or methyl acetate. Six examples were given using lipase P (from *Pseudomonas fluorescens*) and PPL in its free or immobilised form (Fig. 7). The selectivity was the same for the two enzymes, the (S) ester being formed preferentially. Immobilisation of PPL had no effect on the selectivity. In certain cases, namely resolution of (1) and (2) with ethyl acetate as acyl donor, and (5) with methyl propionate as acyl donor a more selective reaction was achieved with PPL. The results indicated in Fig. 7 relate to residual unesterified substrate.

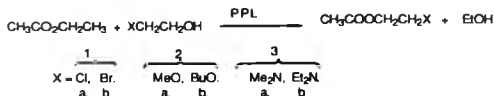
Figure 7. Alcohols resolved using PPL and lipase P (results given for PPL).⁵⁸



* = acylating agent

Another study reported on the effect of electron donating/withdrawing effects of the alcohol substrate in transesterification.⁶⁴

Scheme 5. Transesterification with various alcohols.



Class 1 covers electron withdrawing, 2, electron donating, 3 more strongly electron donating substituents. Moving from a to b in each class gives an increase in steric bulk and in nucleophilicity. Reactions were performed with the acylating agent as solvent. With PPL the less sterically hindered 'a' alcohols showed marked activity, giving 80-90% conversion after 48h. 'b' Alcohols with bulkier substituents, in spite of better nucleophilicity, exhibited a very slow reaction and gave only 26-38% conversion in 48h. This suggests that steric factors are more important than electronic factors in governing lipase activity towards transesterification.

This was also suggested in the following study which considered variation of alcohol and acyl donor⁶⁵ (Scheme 6).

Scheme 6.



R, R¹ = various alkyl groups

Reactions were performed in refluxing THF. Highest yields were obtained with R, R¹ as straight chain alkyl, for example with R=nPr, R¹=n-Pent the ester was obtained in 91% yield.

Introduction of R^1 as an aromatic moiety, $R^1 = \text{PhCH}_2$ or as a moiety containing a double bond (for example $R^1 = \text{CH}_2=\text{CH}-\text{CH}_2-$) also gave a high yield. Introducing either R or R^1 as *i*Pr reduced the yields and as *tert*-Bu eliminated activity altogether. Reactions were much slower with R^1 as *i*Pr or 2-Pent showing possible enantioselectivity, although this was not confirmed.

Transesterification has also been attempted with organometallic substrates⁶⁶ (Scheme 7).

Scheme 7.



R^2 = hexyl or cyclohexyl

R^1 = acetyl, butyryl, capryloyl or lauryl

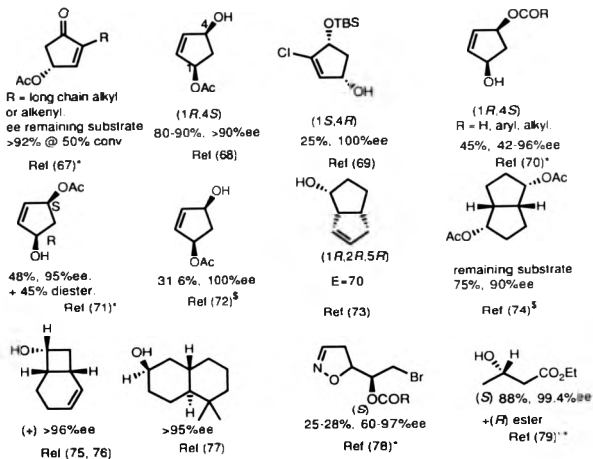
X = H, SnBu_3 , SiMe_3

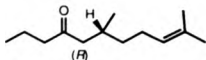
Silyl ethers were poor substrates giving slower reactions than the corresponding alcohol. Stannyl ethers in contrast reacted three fold faster than the corresponding alcohol at a 1M concentration and the effect was larger at higher concentrations. Stannyl ethers of primary alcohols proved to be better substrates than those of secondary alcohols and those of tertiary alcohols did not react at all. Use of activated esters led to faster reaction rates. Equilibrium concentrations were the same irrespective of whether alcohol or stannyl ether were used.

1.2.2 Reactions of secondary alcohols using PPL

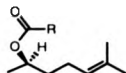
PPL has also been used in resolutions of secondary alcohols as shown in Fig. 8.

Figure 8. Secondary alcohols resolved by PPL-catalysed reactions.

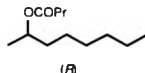




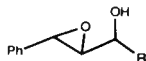
Ref (80)*



(R)-(-)
75%, 90%ee
Ref (81)*

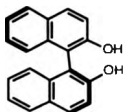


The acyl donor influences
the rate and selectivity
Ref (82)*



(a): R=Et <50% conv., 100%ee
(b): R=Pr 16% conv., 100%ee

Ref (83)



(S)
46% conv., 95%ee
Ref (84)

*=products of an esterification reaction

**= removal of R contaminant from an optically enriched
substrate

§=products of a transesterification reaction.

It is immediately evident that PPL will accept a wide variety of structures and treat them all with high enantiodiscrimination. A detailed study has been made on the effect of temperature, chain length of substrate and enzyme immobilisation on ester formation and enantioselectivity of PPL-catalysed esterification of aliphatic (C5-C10) 2-alkanols and phenylalkanols.⁸⁵ The various substrates were esterified with dodecanoic acid in n-heptane, stirred at defined temperatures for various lengths of time. PPL was found to act preferentially on the *R*-enantiomer in all cases.

Table 1. PPL catalysed esterification of secondary alcohols.⁸⁵

alcohol	T(°C)	conv.(%)	alc. ee(%)	ester ee(%)	E
2-hexanol	40	36	46.1	88.3	27
	70	30	33.8	95.9	71
2-octanol	40	39	60.1	88.0	71
	70	31	41.1	93.1	27
2-decanol	40	27	39.0	94.9	55
	70	34	46.5	93.0	44
1-phenylethanol	40	35	54.8	95.4	71
	70	33	52.7	92.7	42
1-phenyl-1-propanol	40	19	19.9	90.8	26
	70	18	19.6	86.9	17
1-phenyl-2-propanol	40	15	12.7	85.1	14
	70	14	13.6	83.7	13

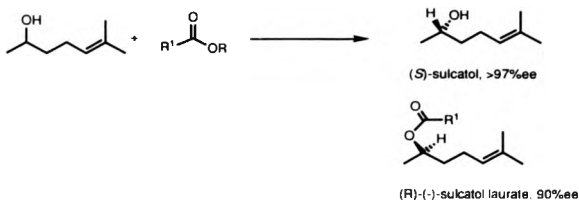
When reactions with 1-phenylethanol and 1-phenyl-1-propanol were performed at 40°C with immobilised enzyme, E values of 392 and 604 respectively were obtained.

At 70°C the enantioselectivity was on average 5.2% higher with 2-alkanols than at 40°C.

Enantioselectivity was increased by the introduction of an aromatic group adjacent to the chiral centre and decreased by increasing the distance between the two. Immobilisation of PPL led to an improvement in enantioselectivity. This was believed to be due to enhancement of the catalytically active surface and removal of water from the enzyme preparation. The esterification of a similar secondary alcohol was performed with

PPL "straight from the bottle" and PPL that had previously been dehydrated under vacuum⁸¹ (Scheme 8).

Scheme 8. Esterification of racemic sulcatol



The enantioselectivity was unaffected by the leaving group (OR) of the ester, and increasing chain length of the acid also had little effect. However, use of the activated ester trifluoroethyl laurate gave a four fold increase in *E* value. A combination of enzyme dehydration and ester selection gave a ten fold increase in the enantiomer ratio and the results obtained under optimised conditions are shown in Scheme 8.

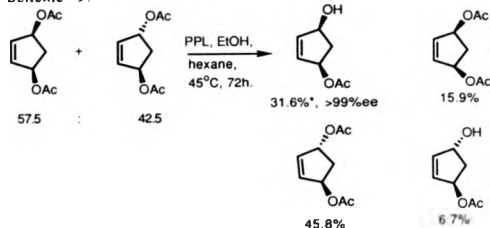
Sulcatol has also been resolved by PPL-catalysed esterification with trichloroethyl butyrate in anhydrous ether.⁸⁰

The selectivity of PPL for secondary alcohols was also exploited in enrichment of an optically enriched product from another reaction.⁷⁹ The (*S*) enantiomer of the desired product was obtained in 90%ee by bakers' yeast reduction of the corresponding ketone. The minor (*R*) isomer was removed by selective esterification using lipase P to gain an increase to 97%ee. A final esterification with PPL under anhydrous conditions gave the (*S*) alcohol in 99.4%ee.

It has also been demonstrated that PPL is selective for the (R) isomer in the esterification of 2-octanol.⁸²

PPL has been used for an interesterification reaction on a mixture of *cis* and *trans* isomers of esters of monocyclic disubstituted diols as shown in Scheme 9.⁷²

Scheme 9.

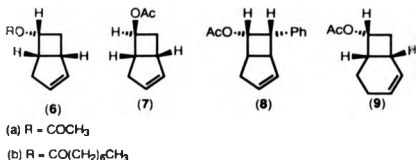


* this yield corresponds to 53% from the *cis* isomer in the substrate.

This example shows that PPL catalyses the reaction of the *cis* isomer much faster than that of the *trans* diacetate. In this reaction PPL gave the mono ester most selectively compared to other enzymes: lipase P for example gave mostly diols.

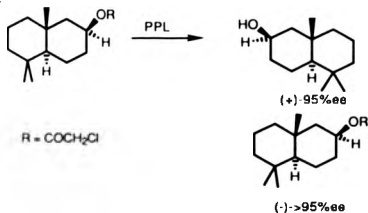
PPL has proved to be sensitive to ring size and steric bulk of substituents in the hydrolysis of racemic esters of bicyclic alcohols.⁷³⁻⁷⁷ The hydrolysis of compounds (6-9) was studied⁷⁵ as shown below (Fig. 9).

Figure 9. Hydrolysis of esters of bicyclic alcohols with PPL



The alcohol from (6a) was obtained in >94%ee. The alcohol from hydrolysis of (7) was obtained in low enantiomeric excess and compound (8) was not hydrolysed at all. In contrast, hydrolysis of *rac*-(9) gave the dextrorotatory alcohol in >96%ee with PPL. The alcohol shown in Scheme 10 was obtained in >95%ee by hydrolysis of an activated ester and by use of a two step procedure.⁷⁷ The product was isolated at 30% conversion and the enriched ester was then further hydrolysed. The opposite enantiomer of the alcohol product was then obtained by chemical hydrolysis of the remaining substrate.

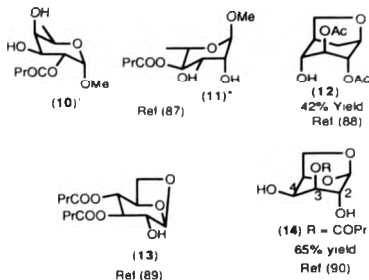
Scheme 10.



1.2.3 Regioselectivity in PPL catalysed reactions.

There are several examples of the use of PPL for the regioselective hydrolysis or transesterification of sugars and their esters. The products of such reactions are shown in Fig. 10.

Figure 10 Products of PPL catalysed hydrolysis of fully esterified sugars.



* products of esterification of non-esterified sugars.

PPL has been shown to hydrolyse selectively the ester from the chemically unreactive C-4 position of 1,6-anhydro-2,3,4-tri-O-acetyl- β -D-glucopyranose to give the 2,3-diacetate (12) in 42% yield after 24h. reaction time. In this example, however the reaction had to be performed at a non-optimum pH to avoid competing non-enzymatic hydrolysis of the C-2 and C-3 acetates.

The butyryl groups of ester (13) were bound much more tightly and reactions could be performed nearer to the pH optimum of the enzyme without fear of acyl migration.⁸⁹ In the PPL-

catalysed hydrolysis of the tri-butanoyl ester the C-2 acyloxy group was hydrolysed preferentially in a solvent system comprising methanol:water 1:4 to give ester (13). Increasing the methanol content to 50% led to almost exclusive deacylation at C-4.

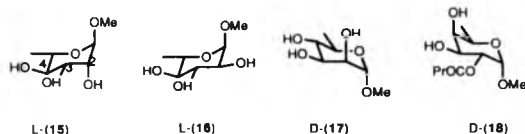
In the chemical hydrolysis of these compounds the selectivity decreased with increasing size of the acyl group but the opposite is true for enzymatic hydrolysis.

In the case of the tributanoyl sugar ester substrate in the reaction leading to monoester (14)(Fig. 10) the ester at C-2 was cleaved first followed by that at C-4. Using the lipase from *Candida cylindracea* the 3,4-di-O-butanoyl derivative was obtained in 90% yield after 29h. reaction time. With PPL the reaction was found to give the 3-O-butanoyl derivative (14) in 65% yield (+ the 4-O-acetyl derivative in 19% yield) after 52h. Similar results were obtained for the corresponding triacetyl ester but with lower yields and selectivity.

PPL was also shown to be selective for the hydrolysis of the primary ester function of peracetylated pyranoses.⁹¹

The acylation of D-(17),(18) and L-(15),(16) hexosides have been compared⁸⁷ (see Fig. 11). The esterifications were performed in THF with 2,2,2-trifluoroethyl butyrate and the extent of conversion with PPL was found to vary widely.

Figure 11. Hexosides acylated by PPL.



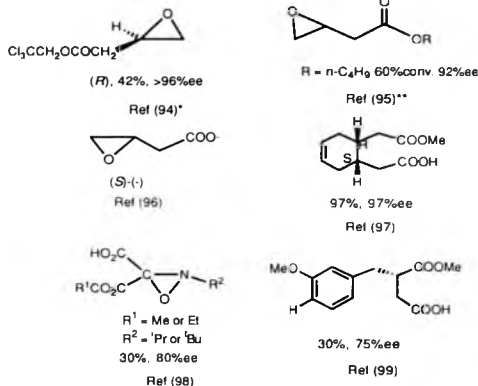
D-Fucopyranoside (18) was the best substrate yielding mostly the 2-O-butyryl ester. Glycosides (15) and (16) were esterified at the 4-position, but the selectivity was greater for L-rhamnopyranoside (15), and was not complete in any example. D-Rhamnopyranoside (17) was recovered almost quantitatively.

PPL was found to acylate the primary hydroxyl groups of glucose, galactose and fructose in pyridine using trichloroethyl butyrate and acetate as acylating agents.⁹² The reaction rate was found to increase with increase in temperature (25-45°C) or concentration of lipase, glucose or acylating agent. Use of less activating esters resulted in a lower reaction rate. PPL has also been utilised in the production of biosurfactants from a sugar alcohol and plant or vegetable oils in reactions performed in dry pyridine.⁹³ The enzyme was kept under vacuum for three days prior to use to lower water content and to increase its stability in organic solvent. PPL was once again selective for primary hydroxyl groups and from the reaction between sorbitol and triolein an equimolar mixture of sorbitol monoesters acylated at C-1 and C-6 was produced.

1.2.4 The use of PPL for resolution of chiral acids.

PPL has also been used for the resolution of a limited number of chiral acids, and these are shown in Fig. 12.

Figure 12. Chiral acids resolved by PPL-mediated reactions.

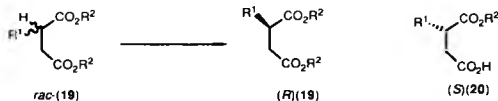


*= product of esterification rather than of hydrolysis

**= remaining substrate, not product.

In one study¹⁰⁰ it was reported that the nature of both acid and alcohol parts of the molecule influenced the enantioselectivity observed (table 2).

Table 2. Influence of acid and alcohol in resolution.



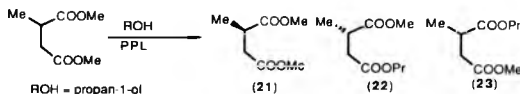
Entry	R ¹	R ²	Remaining yield(%)	ester ee(%)
a	Me	Me	93	95
b	Me	Et	78	75
c	Me	Bu	96	21
d	benzyl	Me	90	98*
e	allyl	Me	80	5
f	n-butyl	Me	85	40
g	NH ₂	Me	90	100
h	NHAc	Me	90	100

* (S)(19) and (R)(20) are the products.

Comparison of entries a,b, and c, shows that higher enantioselectivity is achieved with a short chain alcohol component. Variation of R¹ shows that dimethyl benzylsuccinate (entry d) is an excellent substrate, but it is observed to give a reversal of the selectivity. It should be noted that this reaction is regioselective as well as enantioselective.

Further experiments with this system were performed with the transesterification of dimethyl methylsuccinate.¹⁰¹

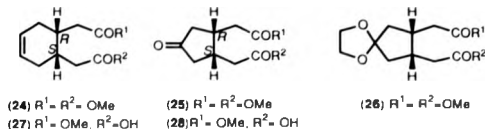
Scheme 11. Transesterification of dimethyl methylsuccinate.



With PPL immobilised on Florisil and using propan-1-ol as both reagent and solvent, (R) ester (21) in >95%ee, together with (S) ester (22) in >95%ee was obtained after 7 days. Compound (23) and the corresponding dipropyl diester were minor products in these reactions.

The effect of the acid part of the structure has been investigated further with the regio- and stereoselective hydrolysis of the following diesters of diacids⁹⁷(Fig. 13).

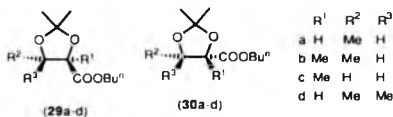
Figure 13. Esters of diacids hydrolysed by PPL.



PPL catalysed the hydrolysis of the (*S*) site of ester (**24**) to give (**27**) in 97% yield and 97%ee. Diester (**25**) was hydrolysed, again at the (*S*) centre to give (**28**) in 48% yield and 6%ee. PPL did not catalyse hydrolysis of ester (**26**).

Further studies were carried out on *cis* and *trans* methyl substituted compounds¹⁰² (Fig. 14).

Figure 14 *Cis* and *trans* dioxolane compounds used as substrates for enzyme-catalysed hydrolyses

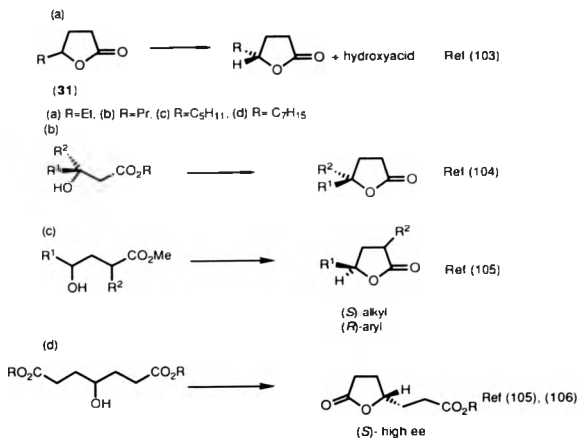


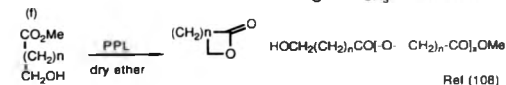
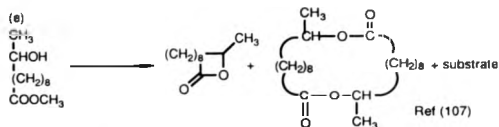
The sensitivity of PPL to steric bulk is demonstrated clearly in this case as only (**29a**) was hydrolysed.

1.2.5 Reactions of lactones.

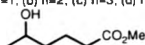
PPL has been utilized for both the enantioselective formation of lactones (intramolecular transesterification) and the enantioselective hydrolysis of these compounds, as shown in Fig.15.

Figure15. PPL-catalysed formation and hydrolysis of lactones.





(32)
 (a) $n=1$, (b) $n=2$, (c) $n=3$, (d) $n=4$



(33)

In the PPL catalysed lactonisation of Fig. 15d the (*S*) lactone was produced in high optical purity. Use of lipase P for this reaction gave the (*R*) lactone in lower ee. For PPL the enantioselectivity was dependent upon the alcohol moiety of the ester. The best results were obtained with the faster reacting ethyl and benzyl esters. The product from the bulky isopropyl ester was obtained in significantly lower ee (46% compared to >98% for the ethyl ester).

Lactone formation versus oligomerisation was studied in the lactonisation of primary hydroxyl esters as shown in Fig. 15f. For (32a,b,d) no lactone formation was observed and for (32c) the average value of X was 6. Hydroxyester (33) underwent a clean lactonisation and the reaction was observed to slow down at 50% indicating that the enzyme was displaying a high degree of stereoselectivity.

In the enantioselective lactonisation of γ -hydroxymethyl esters it was found that the (*S*) isomer reacted 26 times faster than the (*R*) isomer (Fig. 15b). In this experiment both lactone and

recovered hydroxyester were obtained in >94% ee. α -Substituted- γ -hydroxyesters were poor substrates for this reaction.¹⁰⁴

Lactones can also be obtained optically pure by PPL-catalysed hydrolysis¹⁰³ (Fig. 15a). (*S*)-Lactones were hydrolysed preferentially and at 55% conversion remaining (*R*) lactones were obtained in 60-70%ee. The optical purity could be improved by a second enzymatic hydrolysis stopped at 40% conversion.

For (32a,b,d) hydrolyses were performed in 10% CaCl_2 solution at pH7.2. This leads to precipitation of some of the hydroxy acid product and reduces product inhibition.

1.2.6 Conclusions on the use of PPL in organic synthesis.

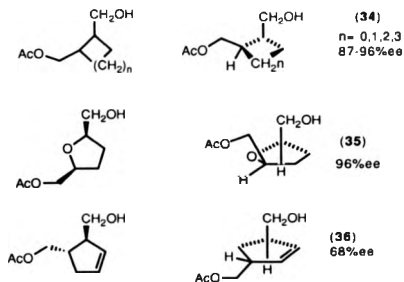
The foregoing discussion illustrates that PPL has been used for reactions of a very diverse range of compounds. These compounds include more primary alcohols than secondary alcohols or chiral acids and there are few examples of the resolution of chiral acids.

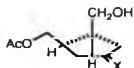
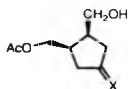
Hydrolysis of prochiral diesters has been demonstrated to give high yields of optically active monoesters with the specificity of the reaction being dependent upon the substituents in the alcohol. Aromatic substrates are readily accepted in resolutions of primary alcohols and have been shown to give high enantioselectivity. In esterification of aliphatic secondary alcohols of varying chain length with PPL the enantioselectivity of the reaction peaked with 2-octanol and decreased with increasing chain length. Introduction of an aromatic moiety

adjacent to the chiral centre gave an increase in enantioselectivity in resolution of short chain alcohols. Cyclic secondary alcohols have also been well resolved using the esterolytic reactions of PPL. In resolution of sulcatol by esterification, increasing chain length of the acid added had little effect upon the enantioselectivity. Fewer examples have been found of PPL-catalysed resolutions of chiral acids. In such examples higher enantioselectivity was achieved using shorter-chained alcohols. By providing a small alcohol moiety that is unlike a natural substrate and is more difficult to hydrolyse, the enzyme is forced to recognise chirality in the acid part of the molecule.

One attempt has been made to establish a model of the active site conformation of PPL based on the results obtained with twelve substrates.⁴⁰ These substrates are shown in Fig. 16, and the proposed model is shown in Fig. 17.

Figure 16. Products of hydrolysis of the corresponding diacetate esters, used to construct the model of PPL.





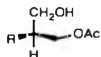
(37)

X = O, $-O(CH_2)_2O-$,
 $-OAc$, $-H$
 $<96\%ee$



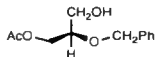
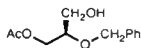
(38)

R = alkyl
 $>95\%ee$



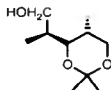
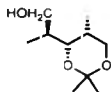
(39)

R = Alkyl, Benzyl,
 Phenyl
 $60 \rightarrow 95\%ee$



(40)

$88\%ee$



(41)

$97\%ee$



(42)

$>97\%ee$



(43)

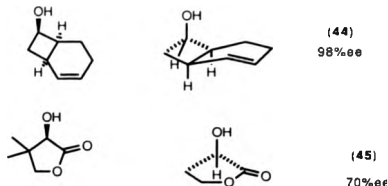
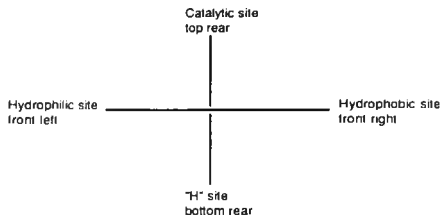


Figure 17. Active site model for PPL.



NB: "H" site = hydrogen site

Four of the compounds (39, 41, 42, 45) clearly do not fit in with this model at all. For the others, no information is available regarding either the binding of each of the enantiomers in the active site, nor the kinetics (k_m , V_{max}) for each of the enantiomers.

Further, it should be noted that these twelve substrates are not representative of the wide range of substrates, the hydrolysis of which is catalysed enantioselectively by PPL. Much more information is required before it can be said with confidence that this model provides an accurate representation of the active site.

1.3 THE LIPASE FROM *CANDIDA CYLINDRACEA*.

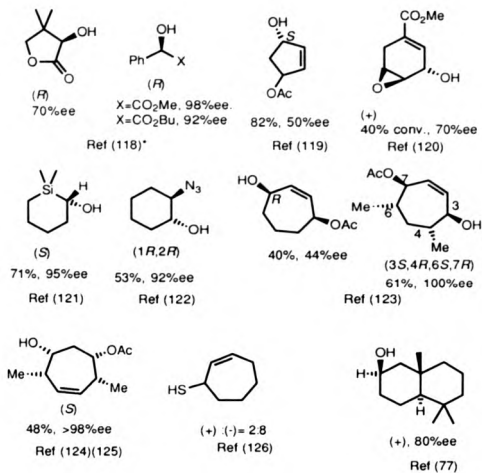
The lipase from the yeast *Candida cylindracea* (recently renamed *Candida rugosa*) (henceforth referred to as CCL) has been utilised in biotransformations almost to the same extent as PPL. The lipase is an exocellular enzyme and, as mentioned in the introduction, its biosynthesis can be increased by addition of certain inducers to the culture medium. Methods for the isolation and purification of the lipase have been published.^{109,110} It has recently been demonstrated that a commercial lipase preparation from Sigma contained several esterases of slightly different ionic state and hydrophobicity.¹¹¹ It has also been demonstrated that upon treatment with deoxycholate and organic solvents (diethyl ether : ethanol 1:1) the enzyme apparently unfolds and refolds to generate a more stable conformer which displays enhanced enantioselectivity.¹¹² The lipase has also been modified with polyethylene glycol to render it soluble and more active in organic solvents and to increase its applicability in biotransformations.^{113,114} It has been suggested that this modification also produces a subtle change in conformation of the active site of the enzyme.¹¹⁵ CCL has been reported to be active in freons as solvents¹¹⁶ and in reverse micelles although in the latter the enzyme was strongly deactivated compared to the lipase from *Rhizopus delemar*.¹¹⁷

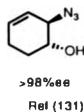
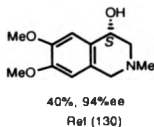
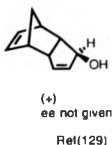
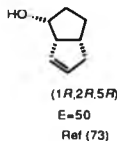
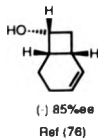
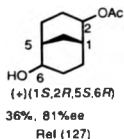
The use of CCL in biotransformations appear to be largely for reactions of secondary alcohols and there are very few reported uses of CCL in the resolutions of primary alcohols.

1.3.1 Resolutions of secondary alcohols

A great many examples are available in the literature of CCL-catalysed resolutions of cyclic secondary alcohols and a number of these are shown in the figure below.

Figure 18. Cyclic secondary alcohols resolved via CCL-catalysed reactions.

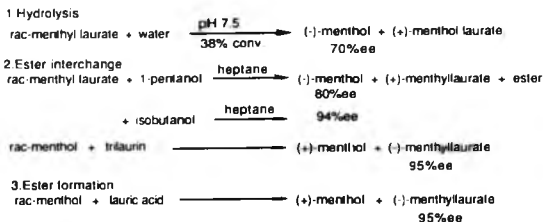




CCL has been shown to display complete enantiodiscrimination in esterolytic reactions of menthol and its esters and so this compound has been used to optimise reaction conditions for various hydrolytic and esterification procedures.

Four methods for resolution have been compared.¹³²

Scheme 12. Resolution methods used with CCL.

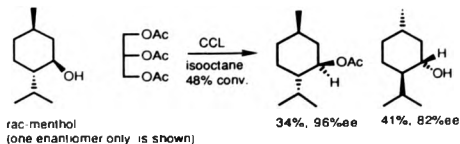


The highest initial reaction rate was that of hydrolysis but owing to product inhibition the desired conversion could not be

attained. Ester interchange reactions starting from menthyl laurate were extremely slow. Ester formation reactions in either trilaurin or lauric acid occurred at similar rates. All reactions in organic solvent gave products of high optical purity. This increase in selectivity for reactions in organic solvent was believed to be due to the higher conformational rigidity of the enzyme under such conditions.¹³²

In another report the interesterification of (\pm)-menthol with triacetin (Scheme 13) was compared to the hydrolysis of (\pm)-menthyl acetate and esterification of (\pm)-menthol with acetic acid.¹³³

Scheme 13.



It was found that this interesterification reaction reached its maximum rate at 90°C whereas in hydrolysis the enzyme lost activity at 50°C and little esterification activity was detected at 90°C.

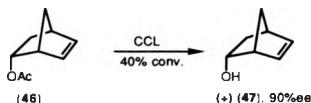
The interesterification rate was reduced by addition of water but with a water content of <3% the enzyme showed neither interesterification nor esterification activity. With the enzyme immobilised maximum interesterification activity was achieved with 2% water. This led to the conclusion that the native structure of the enzyme was stabilised by immobilisation.

The interesterification activity was increased by the use of longer chain triglycerides, but enantioselectivity was dramatically reduced with substrates such as trilinolein. The effect of acyl donor on the esterification reaction has also been studied.¹³⁴ The optical purity and yields of the menthol ester were compared with hexanoic, lauric and phenylvaleric acids as acyl donor. Both yield and optical purity were dependent upon the acyl donor showing that the physical properties of the acyl donor have a significant influence on the equilibrium constant.

In the same study¹³⁴ the E values obtained for a reaction in phosphate buffer and in water-saturated isooctane differed, showing that the enzyme was undergoing different conformational changes in different media. Data were presented for the dependence of optical purity of product and remaining substrate on the degree of conversion for the formation of hexanoate, laurate and 5-phenylvalerate esters of L-menthol. The best combination of selectivity and reactivity was achieved with 5-phenylvaleric acid. This was confirmed by Japanese workers who found that while long chain fatty acids were excellent acyl donors low selectivity was observed for L-menthol.¹³⁵ Short-chain fatty acids were poor substrates but offered high enantioselectivity. 5-Phenylvaleric acid proved to be a good compromise on both counts.^{135,136} The enzyme was immobilised in a urethane prepolymer for these reactions and non-polar solvents were favoured. Reaction with other terpenes was investigated. Gel-entrapped CCL gave esters of borneol and citronellol with oleic acid as acyl donor but linalool, nerolidol and α -terpineol were not substrates.

The Austrian group of Faber and his co-workers have been concentrating on CCL-catalysed reactions of bicyclic esters. It was found immediately that bicyclic esters with a norbornane-type structure were hydrolysed by few enzymes. A screening experiment on the hydrolysis of the ester (46) established that only CCL would perform the desired hydrolysis¹³⁷.

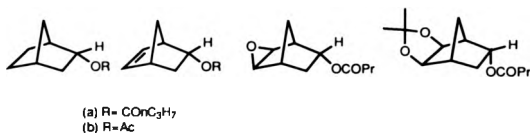
Scheme 14



It was also discovered that *endo* substrates (for example (46)) gave a high degree of enantioselectivity but that their *exo* counterparts were hydrolysed with low to moderate ee (CCL) or not at all (lipase P).

Two reports^{139,140} assessed the ring substitution pattern required for high stereoselectivity. The following compounds were hydrolysed with high selectivity. The *R* centre was invariably hydrolysed (Fig. 19).

Figure 19. Compounds hydrolysed with high selectivity.



A further report elucidated the bridgehead requirements for high enantioselectivity using the following substrates (Fig. 20).

Figure 20. Elucidation of the bridgehead requirements for a selective reaction.



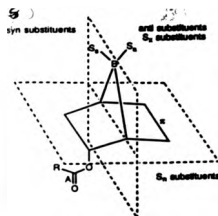
$X = H$ or $COPr$

$R^1, R^2 = H$ or $R^1, R^2 = \text{double bond}$

$R^3, R^4 = \text{ester or methoxy}$

Again *endo* esters with the *R* configuration centre were cleaved preferentially. It was found that esters with pi electrons in the 5-6 position gave better results than the corresponding saturated compounds. Increasing the steric hindrance at bridgehead C-7 dramatically decreases the enantioselectivity. Compound (50) gave the best results. From this information a general substrate model was presented in a further report¹⁴¹ (Fig. 21).

Figure 21. General substrate model for the hydrolysis of norbornane-type esters by CCL .

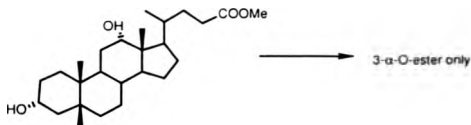


REGION

- A Ester had endo configuration for high enantioselectivity with (R) configuration cleaved preferentially. Exo derivatives showed low enantioselectivity. Increasing chain length to octanoate increased rate of reaction.
- B May contain hetero atom (O) but must be small. Enantiomeric ratio is roughly halved going from bicyclo[2.2.1]heptane to bicyclo[2.2.2]octane.
- Sa,Ss In keeping with the above the substituents must be small and steric requirement should be kept to a minimum
- Sx Substituents may be large with bulky group giving slight increase in enantioselectivity and slight decrease in rate of reaction.
- Sn Should not be occupied or if so substituent should be small
- x site x electrons in this site encourage enantioselectivity.

The variety of cyclic secondary alcohols resolved using CCL however shows that the substrate specificity of this enzyme is very broad. Two examples have been found of selective reactions on steroids. In one study¹⁴² the 3 α -O-ester was formed exclusively from reaction of a polyhydroxysteroid with trichloroethyl butyrate in anhydrous benzene catalysed by CCL (Scheme 15).

Scheme 15. Esterification of a steroid.



This reaction could be performed on a preparative (0.5g) scale giving a 70-85% yield.

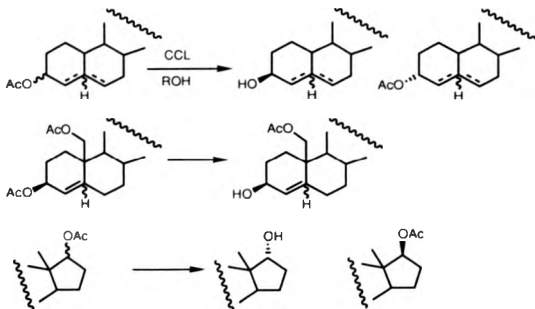
Further steroid substrates were used to elucidate the structural preferences of the enzyme. CCL exhibited a wide substrate specificity and in each reaction a single product was formed. However this enzyme was inactive towards the 12 α -OH, 7 α -OH

and 7 β -OH positions. Sensitivity to the steroid side chain was investigated briefly and bulky compounds were readily accepted for esterification.

Attempts to hydrolyse steroid acetates were unsuccessful.¹⁴² The authors suggest that this is due to the inability to form an acyl-enzyme intermediate.

Another earlier study indicated however, that steroid esters could engage in a transesterification reaction with 1-octanol.¹⁴³ The following reactions were observed (Scheme 16)

Scheme 16. Hydrolysis of steroid esters via transesterification with octanol (steroid substructures only are shown).

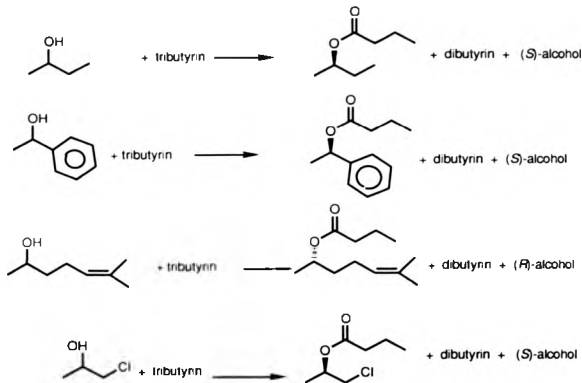


It was found that 3 β -esters were hydrolysed in high yields but 3 α -esters were unaffected. 17 β -Esters resisted cleavage. With 3,17-diacetoxyestradiol the acetoxy group at the 17 α position was hydrolysed more slowly than the acetoxy group at position 3. Both ester groups in 26-hydroxycholesterol diacetate were hydrolysed. 21-Acetoxycorticosteroids were cleaved and allylic

esters were cleaved with no side reactions, but the 19-acetoxy esters resisted cleavage.

Some straight chain secondary alcohols have also been resolved using CCL. A variety of secondary alcohols have been resolved *via* transesterification with tributyrin. CCL was placed in a biphasic system where the aqueous phase was a solution of the enzyme in water confined to the pores of chromosorb and the organic phase was a solution of an alcohol in tributyrin.¹⁴⁴ Under these conditions CCL accepted a wide variety of alcohols in transesterification and with chiral alcohols a marked stereo selectivity was displayed (Scheme 17).

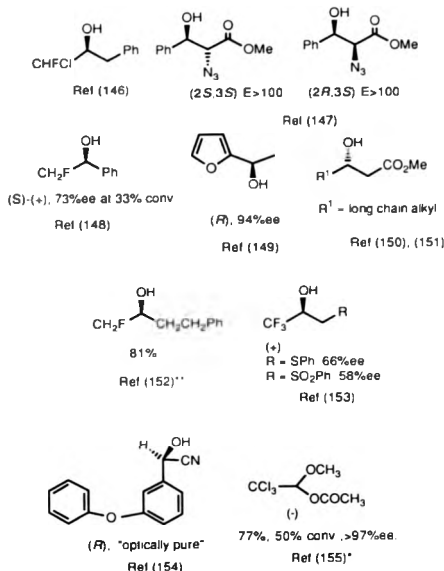
Scheme 17 Acyclic secondary alcohols resolved using CCL¹⁴⁴



CCL was selective for the *R* enantiomer. The enantiomeric excess in each case was >90% for both ester and recovered

alcohol. Alcohols successfully resolved include 2-butanol, 2-octanol, secondary phenethylalcohol and 6-methyl 5-hepten-2-ol.¹⁴⁵ Other examples of secondary alcohols resolved using CCL are shown in Fig. 22.

Figure 22. Secondary alcohols resolved using CCL

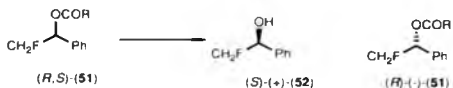


* = recovered substrate

** It is not clear from the paper which isomer is hydrolysed - it may be deduced that the (S)-(+ product is formed from work on related compounds described in reference 148.

Comparison of the following examples demonstrates the influence that the acid moiety can have upon the selectivity of the hydrolysis¹⁴⁸.

Table 3. Influence of the acid moiety in resolution of a secondary alcohol



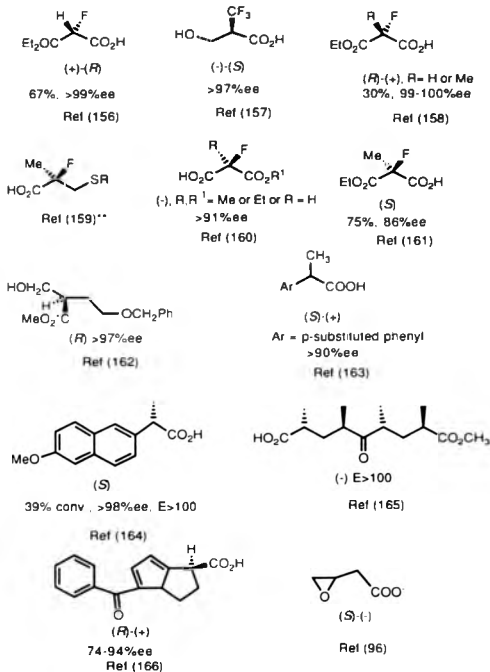
R	hydrolysis ratio(%)	product ee(%)
CH ₃	3.4	2.6
n-C ₃ H ₇	4.7	1.7
i-C ₃ H ₇	3.3	7.3
n-C ₄ H ₉	4.6	5.2
n-C ₅ H ₁₁	3.1	3.1
n-C ₇ H ₁₃	4.6	2.5
cyclo-C ₆ H ₁₁	1.1	7
Ph	2.8	6.3

The fact that low optical purities were obtained in hydrolyses of esters with longer chain acid components and that these values were found to increase with the isopropyl and phenyl substituents suggests that CCL prefers a bulky acid component.

1.3.2 Esterolytic reactions with chiral acids.

CCL has also been used in the resolutions of a large number of chiral acids and some examples of these are shown in Fig.23.

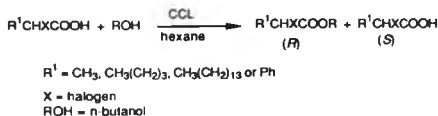
Figure 23. Chiral acids resolved using CCL.



**=the reaction was simply used as a mild hydrolysis without causing racemisation.

Klibanov *et al*¹⁶⁷ have reported the resolution of a wide variety of acids via an esterification reaction (Scheme 18)

Scheme 18. Resolution of chiral acids.

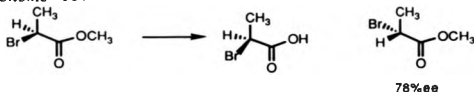


The conversion was adjusted to obtain either the optically active ester or optically active acid and either could be obtained with >90% ee.

CCL was tolerant to the nature of the group R^1 . Both alkyl and aromatic groups, large and small were accepted. However the electron-withdrawing halogen was essential. Acids in which X was alkyl or hydroxyl were unreactive. The same selectivity was observed in resolutions of a similar series of carboxylic acids by a hydrolytic procedure.¹⁶⁸

The reactions were performed in a two phase mixture of salt-saturated water with carbon tetrachloride or perchloroethylene. The product acid is soluble in the aqueous phase and the organic phase becomes enriched in the L-ester. Ten examples were given of which one is presented in the scheme below.

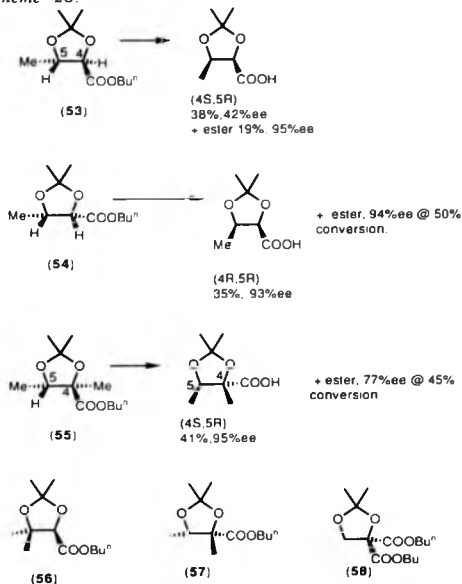
Scheme 19.



This process was modified to avoid the use of large amounts of water and yet avoid inhibition of CCL by the product.¹⁶⁹

In the resolution of a cyclic substrate CCL was discovered to be sensitive to steric influences¹⁰² (Scheme 20).

Scheme 20.



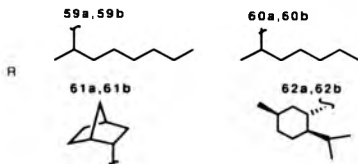
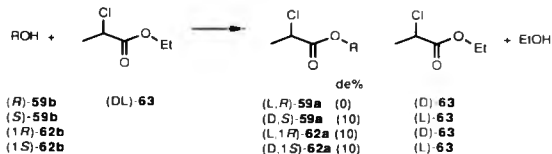
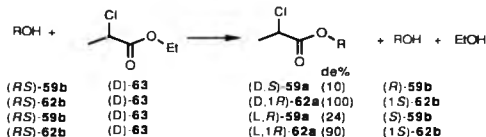
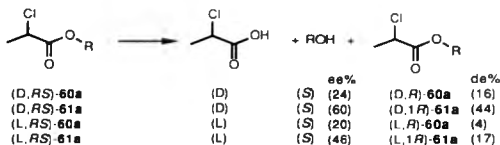
Rac-(56) and (57) were not hydrolysed by either PPL or CCL and *rac*-(58) was hydrolysed but with very low selectivity. The *cis* isomer was found to react considerably more slowly than the *trans* isomer (54 cf 53) and with an additional 4-methyl group only the *trans* isomer (55) is a substrate. A very selective hydrolysis is seen in this case.

The enantioselectivity of hydrolysis of the *cis* and *trans* isomers differs as regards to C-4 and is apparently determined by C-5. Without the 5-methyl group the enantioselectivity drops (58) and with 5,5 disubstitution the ester is no longer a substrate (56).

The dependence of the enantioselectivity of CCL-catalysed reactions on acid or alcohol components of the compound has been assessed with diastereomeric esters.¹⁷⁰ The lipase-catalysed hydrolysis of (D)- and (L)-2-chloropropanoates of four racemic alcohols, transesterification of (D)- and (L)-2-chloropropanoates with racemic alcohols and transesterification of ethyl (DL)-2-chloropropanoate with optically pure alcohols was examined (Scheme 21).

Scheme 21 Dependence of enantioselectivity on acid and alcohol components.

Note that to avoid confusion the chirality of the ester and alcohol respectively are given as D-, L- and R, S



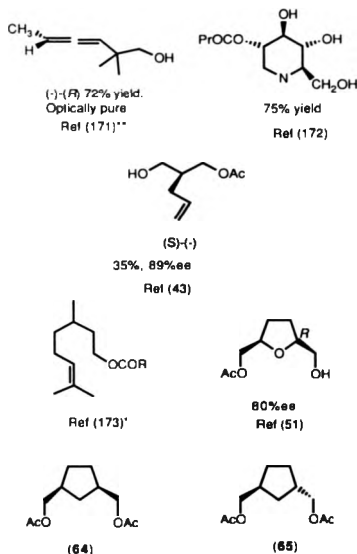
The results obtained from such experiments suggested that in hydrolytic reactions careful choice of an additional centre of chirality present in the acid part of an ester could improve selectivity of enzymatic resolution of a racemic alcohol. However, resolution of racemic acids was not improved by the introduction of chirality into the alcohol component of an ester

substrate. Corresponding results were obtained with transesterifications. It seemed apparent that the enzyme preferentially recognised chirality in the alcohol rather than the acid component of the ester substrates.

1.3.3 Resolution of primary alcohols.

CCL has been little used for the resolution of primary alcohols but some examples of this are shown in Fig. 24.

Figure 24. Primary alcohols resolved via CCL catalysed reactions.




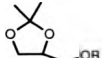
* = product of esterification rather than of hydrolysis

** = product of transesterification; optical purity was not defined.

It is interesting to note that the enzyme recognised axial chirality albeit with low selectivity.¹⁷¹ Laumen *et al* have demonstrated that in the hydrolysis of diacetate (64) the selectivity of CCL is the reverse of that noted previously with PPL,⁵¹ and hydrolysis of the corresponding *trans* diacetate (65) gave very low selectivity (Fig. 25).

It has been reported¹⁷³ that longer chain fatty acids are excellent acyl donors compared to short chain compounds. In the same study pivalic acid and 3-phenylpropionic acid were non-substrates, presumably due to steric hindrance. The effect of acylating agent has also been studied in the transesterification of glycerol acetonide with a variety of acylating agents.⁴⁸ Results are given below (Table 4)

Table 4. Study of the effect of acylating agent on the enantioselectivity of CCL-catalysed esterifications.

<div style="display: flex; align-items: center; justify-content: center;"><div style="text-align: center;"></div><div style="margin: 0 10px; text-align: center;">CCL, enol ester ————— organic solvent</div><div style="text-align: center;"></div></div>		
ester	rel. rate	E
ethyl acetate	1	1.2
isopropenyl acetate	2.0	1.4
isopropenyl valerate	8	2.7
vinyl acetate	62	1.4
vinyl propionate	122	2.0
vinyl valerate	13	3.1

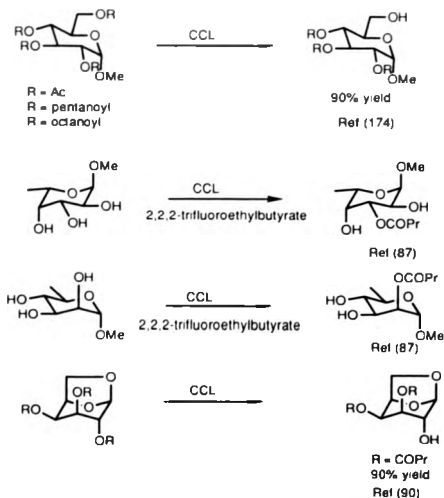
It may be seen that the acylating ester affects both rate and selectivity of the reaction, with the longer chained valerate giving higher selectivity.

The organic solvent was also varied and this was found to influence rate but not selectivity. Methyl β -D-glucopyranoside was esterified with vinyl acetate to give exclusively the primary acylated product.

1.3.4 Regioselective Reactions of Carbohydrate Substrates

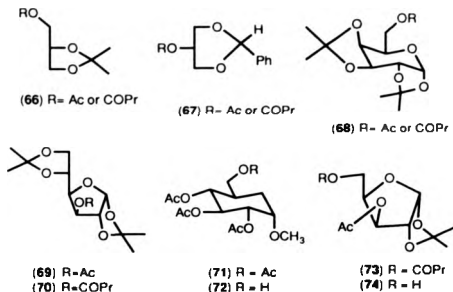
Fig 25 shows CCL-catalysed reactions of sugars and sugar esters.

Figure 25 CCL-catalysed reactions of sugars



It has been reported that CCL was selective for acylation at the primary position.¹⁷⁵ CCL has been compared with PPL in the removal of protecting groups on glycerides and glycosides.¹⁷⁶ CCL hydrolysed both the primary and secondary n-butyrate and acetate functions of compounds (66-68) whereas PPL hydrolysed only the primary butyrates of (66) and (68). CCL hydrolysed (70) and not (69) whereas PPL hydrolysed both. CCL effected complete regioselective hydrolysis of (71) to give (72) in a mixture of buffer and di-n-butyl ether. For compound (73) PPL gave nonselective hydrolysis of both acyl groups whereas CCL gave rapid conversion to (74)(Fig. 26).

Figure 26. Compounds deprotected using PPL and CCL.



CCL has also been used in the nonselective hydrolysis¹⁷⁷ and selective hydrolysis¹⁷⁸ of enol acetates and has been used in amidation reactions.^{179,180}

Concluding comments.

The clear trend that has appeared in the reactions of CCL is that it has been applied most successfully to various reactions of secondary alcohols.

It has also been used in resolutions of chiral acids but has seen little use in reactions of primary alcohols.

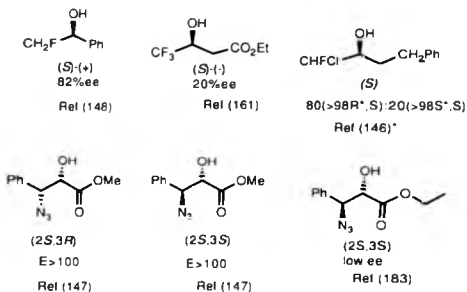
1.4 LIPASE P FROM *PSEUDOMONAS FLUORESCENS*

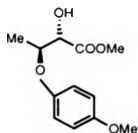
1.4.1 Lipase P-catalysed reactions on secondary alcohols.

The most immediately striking feature of reactions catalysed by lipase P is that most of them consist of the resolution of chiral secondary alcohols, either by hydrolysis of the racemic ester or by esterification of the racemic alcohol.

In Fig. 27 are shown a number of non-cyclic alcohols which have been resolved with lipase P by hydrolysis of a racemic ester, with varying degrees of success.

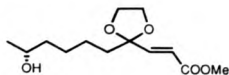
Figure 27 Secondary alcohols resolved with lipase P





(2*S*,3*S*) 70%ee
+ ester, 95%ee

Ref (184)



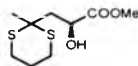
(*R*) 42% conv >99%ee

Ref (185)



X = Cl, Br
>99%ee

Ref (186)



26%, 96%ee

Ref (187)



(*R*)

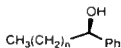
R = CH₃, Ph, PhCH₂CH₂, PhCH=CH-
>80%ee @ 40% conv

Ref (188)



R = octyl, (*S*)-(-) 72%ee
R = PhCH₂CH₂, (*S*) 89%ee

Ref (189)



n = 5, 6, 7

(*R*)-(+), 90-100%ee

Ref (190)



90-92%ee

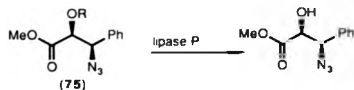
Ref (191)

* product of esterification rather than hydrolysis.

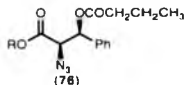
These alcohols were generally resolved *via* hydrolysis of either an acetate or a butyrate ester.

A variety of acyl chains were used to investigate the effect of variation of the acid component in the hydrolysis of esters of a chiral alcohol.¹⁴⁷

Table 5. Investigation of the effect of variation of the acid component in the hydrolysis of esters of a chiral alcohol.



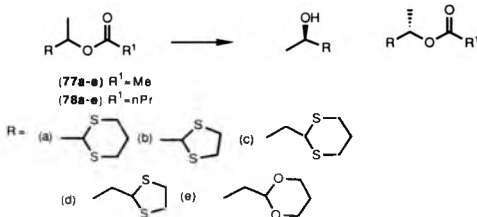
Ester	conv. (%)	ee (%)	E
butanoate	40	98	100
acetate	40	97	100
chloroacetate	40	86	13
isobutyrate	40	98	100
cyclohexylcarboxylate	27	93	39
octanoate	40	85	12



Chemical hydrolysis was found to occur to a high degree with the chloroacetate with a concomitant reduction in ee. The isobutyrate and cyclohexylcarboxylate esters showed very slow rates of reaction. The octanoate showed a reasonable reaction rate but this was lower than with the acetate and butyrate. Butyrates were utilized for further experiments on this system. It is interesting to note that lipase P was not active on the closely related compound (76). The authors suggest that lipase P needs two chemically activating groups vicinal to the group to be hydrolysed, namely the azide and carbonyl groups. This is evident in compound (75) above but not in compound (76) as the phenyl group is not sufficiently activating.

Acetate and butyrate esters (**77a-e** and **78a-e**) were hydrolysed with lipase P¹⁹³ (Scheme 22). No appreciable difference in selectivity was observed upon changing the acid component of the esters.

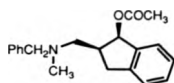
Scheme 22.



In the examples of (**77a-d**, **78a-d**) the (*R*) alcohol was produced with high enantioselectivity. For (**77e**, **78e**) the selectivity was reversed and was dramatically reduced (at 40% conversion the (*S*) alcohol was obtained in 26%ee).

Similar alcohols have been resolved *via* esterification or transesterification reactions. Examples are shown in Fig. 28.

Figure 28. Products of esterification reactions of secondary alcohols using lipase P.



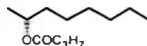
(-) 43%, 97%ee

Ref (194)



X = halogen
>90%ee

Ref (195)



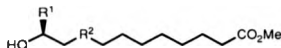
(R) 73%, 72%ee
+ alcohol 70%, 99%ee

Ref (196)



R = ClCH₂ (S) E = 24
R = C₄H₉ (R) E = 26

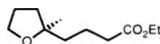
Ref (197)



R¹ = H, Me, C₆H₁₃
R² = CH₂CH₂, CH=CH (E or Z)

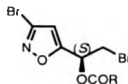
(R)-product, >98%ee

Ref (198)*



(R) <32%ee

Ref (106)**



R = octanoate
30% conv 97%ee
Ref (78)

*substrate for lactonisation

** product of lactonisation of a hydroxy ester.

In the following example¹⁹⁷ the esterification was performed with isopropenyl acetate on substrates of various chain length, as shown in Table 6.

Table 6. Esterification of various secondary alcohols catalysed by lipase P.

$ \begin{array}{c} \text{OH} \\ \\ \text{R}-\text{CH}-\text{CH}_2-\text{OTs} \end{array} \xrightarrow[\text{isopropenyl acetate}]{\text{Lipase, hexane}} \begin{array}{c} \text{OCOCH}_3 \\ \\ \text{R}-\text{CH}-\text{CH}_2-\text{OTs} \end{array} \quad \begin{array}{c} \text{OH} \\ \\ \text{R}-\text{CH}-\text{CH}_2-\text{OTs} \end{array} $					
R	Pref	Time(h)	conv(%)	P,ee(%)	S,ee(%)
ClCH_2	S	68	47	83	75
$n\text{-C}_4\text{H}_9$	R	92	43	86	66
$n\text{-C}_{10}\text{H}_{21}$	R	116	30	87	37

P = product, S = remaining substrate.

Thus it appears that when $\text{R}=\text{C}_{10}\text{H}_{21}$ the substrate has difficulty fitting into the active site and that the position within the active site is reversed for $\text{R} = \text{ClCH}_2$ and $n\text{-C}_4\text{H}_9$.

The enantioselectivity of this system was improved by performing a transesterification reaction (Table 7).

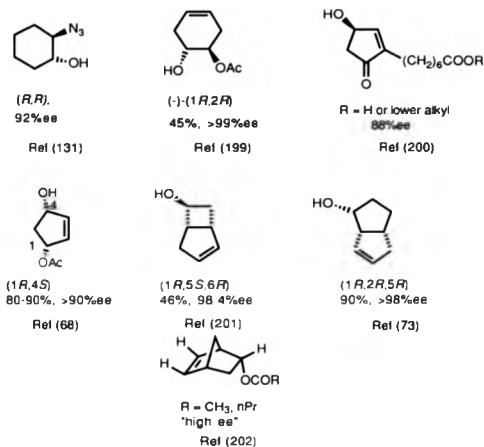
Table 7. Transesterification of esters of secondary alcohols catalysed by lipase P

$ \begin{array}{c} \text{OCOCH}_3\text{H}_7 \\ \\ \text{R}-\text{CH}-\text{CH}_2-\text{OTs} \end{array} \xrightarrow[\text{lipase, hexane}]{\text{BuOH} \quad \text{PrCOOBu}} \begin{array}{c} \text{OH} \\ \\ \text{R}-\text{CH}-\text{CH}_2-\text{OTs} \end{array} \quad \begin{array}{c} \text{OCOCH}_3\text{H}_7 \\ \\ \text{R}-\text{CH}-\text{CH}_2-\text{OTs} \end{array} $					
R	Pref	Time(h)	conv(%)	P,ee(%)	S,ee(%)
ClCH_2	S	96	50	96	96
$n\text{C}_4\text{H}_9$	R	120	25	90	31
$n\text{C}_{10}\text{H}_{21}$	R	120	26	>98	34

P = product, S = remaining substrate.

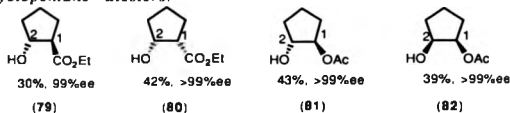
Further studies with lipase P have concentrated on cyclic secondary alcohols, in both hydrolysis and esterification reactions. In Fig. 29 are shown a number of products from such reactions.

Figure 29. Cyclic secondary alcohols resolved with lipase *P*.



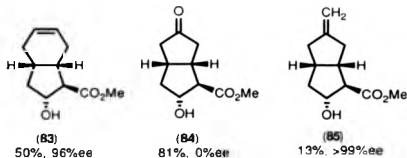
Detailed studies have been reported on the lipase *P*-catalysed hydrolyses of cyclopentane 1,2-diacetates.²⁰³ The results presented in Fig. 30 show that lipase *P* will hydrolyse the acetate from the (*R*) centre (C-2) with high selectivity apart from in the case of compound (82) when the (2*S*) alcohol centre was produced.

Figure 30. Products of lipase *P*-catalysed hydrolysis of cyclopentane diesters.



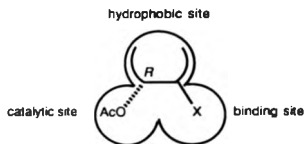
This information was extended to 6- and 7- membered ring diacetox compounds.²⁰⁴ Results showed that the *R* centre was hydrolysed preferentially with high enantioselectivity, regardless of ring size. In addition to this the highly enantioselective hydrolysis of bicyclo [4.3.0.]non-3-en-8-yl acetate (**83**) and non-selective hydrolysis of bicyclo[3.3.0.]octan-3-yl acetate with a carbonyl function (**84**)²⁰³ suggested that the presence of a hydrophobic group on one ring and the acetate on the other ring leads to selectivity. This was confirmed by hydrolysis of ester (**85**) with high enantioselectivity.

Figure 31. Bicyclic esters hydrolysed by lipase P



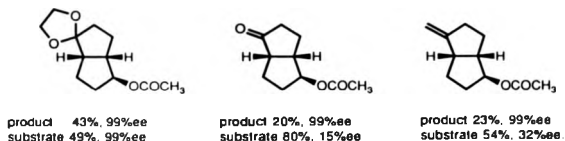
On the basis of these results a three site model was tentatively suggested for the active site of lipase P.²⁰⁴ Alkoxy carbonyl or carboxylate groups were required adjacent to the acetox function to occupy a binding site and in bicyclic systems the ring not containing the acetate function should be hydrophobic. Fig. 32 shows the model proposed.

Figure 32. Active site model for lipase P.



This model has recently been modified following the observation that *exo* substrates are hydrolysed with high selectivity²⁰⁵ (Fig. 33). The corresponding *endo* compounds were resistant to hydrolysis.

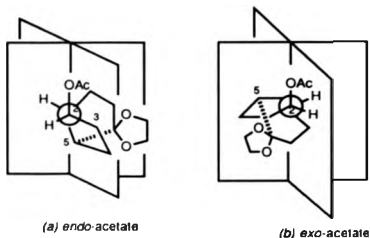
Figure 33. *Exo*- substrates hydrolysed with high selectivity using lipase P.



A stereochemical analysis of the substrate was performed to explain these observations. The substrates were drawn in the Newman projection. The bicyclic skeleton of the (1*R*, 2*R*, 5*S*) enantiomer of the *endo* substrate was found to occupy the right hand segment, as shown in Fig. 34a, whereas its enantiomer occupied the left hand segment. Both right and left hand segments were occupied in the case of the (1*S*, 2*S*, 5*S*) *exo* acetate (Fig. 34b) and its (1*R*, 2*R*, 5*R*) enantiomer. It was

concluded that active substrates were bound only via the right hand segment as in Fig. 34a.

Figure 34. Newman projections of *endo* and *exo* substrates.

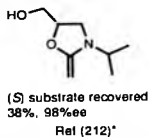
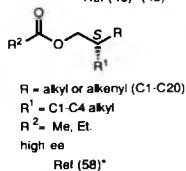
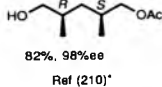
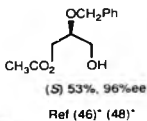
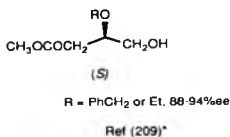
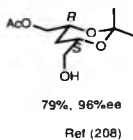
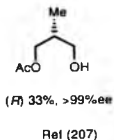
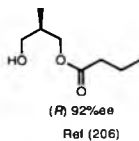
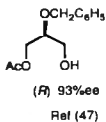


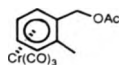
It was proposed that the active site of lipase P has two pockets- a catalytic/binding site (this can serve as both since the ester or acetate adjacent to the hydrolysed function has no influence on the stereochemistry) and a hydrophobic site.

1.4.2 Esterolytic reactions of lipase P on primary alcohols.

Lipase P has also been utilised in the resolution of primary alcohols.

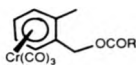
Figure 35. Primary alcohols resolved by lipase *P*-catalysed reactions.





(S) 48%, 98%ee

Ref (213)*



R = Me, nC₇H₁₅, nC₁₅H₃₁, Ph
>88%ee

Ref (214)*

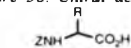
* = products of esterification reactions rather than of hydrolysis.

The activities and selectivities of lipase P and PPL have been compared⁵⁸ in reactions with primary alcohols as described in section 1.2. The two enzymes were found to display similar selectivities although almost without exception lipase P gave higher enantiomeric purities. Similar selectivity was demonstrated by these enzymes upon glycerol derivatives.⁴⁶

1.4.3 Resolutions of chiral acids.

There are also several examples of lipase P being used in the resolution of chiral acids as shown in Fig. 36.

Figure 36. Chiral acids resolved by lipase P

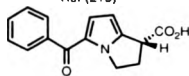


L-isomer

R = hexyl

87%ee

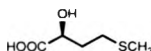
Ref (215)



(R)-(+)-(87)

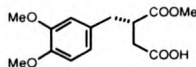
74-94%ee

Ref (166)



(S) 42%, >98%ee

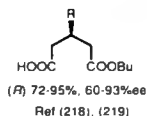
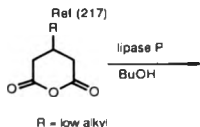
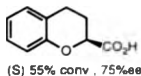
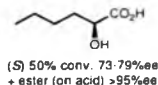
Ref (216)



(86)

40% conv., 40%, >95%ee

Ref (99)



Lipase P has also been used in the hydrolysis of lactones,²²⁰ and in the regioselective esterification and hydrolysis of nucleosides and their esters respectively.^{221,222}

Again, comparing the activities of lipase P and PPL it was found that only lipase P would catalyse the hydrolysis of the corresponding dimethyl diester to give the acid (86).⁹⁹ These two lipases have been demonstrated to have opposite selectivities in hydrolysis of methyl and ethyl esters of acid (87) (Fig. 36). Lipase P gave the (R)-(+)- acid in high ee, whereas PPL gave the (S)-(-) enantiomer in low ee.

1.4.4 Conclusions.

The preceding examples show that lipase P can exhibit extremely high enantioselectivity in the resolutions of both cyclic and noncyclic secondary alcohols. The structures in Fig. 20 generally have one substituent adjacent to the chiral centre that is no larger than a methoxy group (Me or MeCOO or CF₃) and a second which typically contains an aromatic ring or a long chained alkyl group. Thus it appears that for the resolution of secondary alcohols with a substantial difference in the size of the substituents lipase P may be the preferred enzyme. In the resolution of cyclic alcohols the model for the active site as

proposed by Xie and co-workers²⁰⁵ seems to predict the likely outcome of events. Monocyclic diesters and bicyclic diesters with the acetate on one ring and the other ring being essentially hydrophobic are hydrolysed with a high degree of selectivity. Although fewer primary alcohols have been resolved with lipase P some excellent results have been achieved in, for example, the resolution of glycerol esters and organometallic substrates. Relatively little work has been published on the resolution of chiral acids with lipase P.

1.5 OTHER MICROBIAL LIPASES.

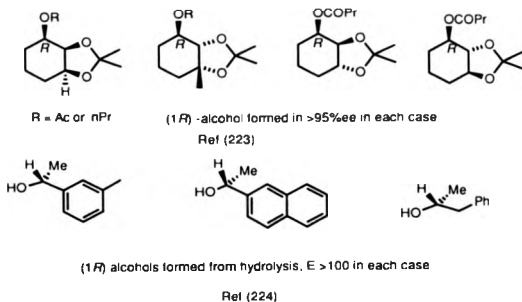
Many microbial lipases are available commercially and have been used for certain applications. The reactions of some of these enzymes are discussed below.

1.5.1 Lipase SAM II from *Pseudomonas*.

This enzyme is stated to be a purified form of lipase P from *Pseudomonas* sp. and the results described for this enzyme should be considered together with those described above for lipase P.

Fig. 37 shows compounds resolved using microbial ester hydrolase SAM II from *Pseudomonas* sp.

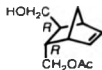
Figure 37. Compounds resolved using lipase SAM II.





>98%ee, E 700

Ref (225)



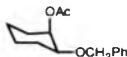
23.5% conv., 96%ee

Ref (227)



R = Ph, PhCH₂, PhO, PhCH₂O
(1R) alcohols, E>100 in each case

Ref (226)*



* products of irreversible transesterification reactions.

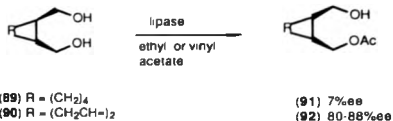
As with lipase P esters hydrolysed were acetates or butyrates. Almost without exception E values were exceedingly high for this enzyme with these substrates. Notable exceptions are found with hydrolyses of diesters (**88a,b**) when (**88a**) was a nonsubstrate and (**88b**) was hydrolysed with low enantioselectivity. (1R,2S)-Products were obtained essentially enantiomerically pure from hydrolysis of (**88c-e**)

With the transesterification of diols (**89**) and (**90**) reactions with ethyl acetate were very slow and reaction with vinyl acetate was always accompanied by the formation of large amounts of diacetates.

Scheme 23.

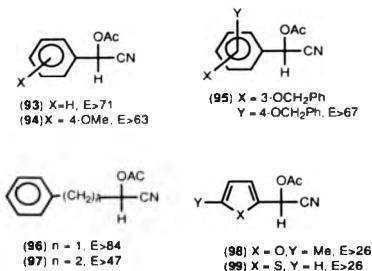


- (a) R = C(CH₃)₂
- (b) R = (CH₂)₄
- (c) R = (CH₂)₂
- (d) R = (CH₂)₃
- (e) R = (CH₂CH=)₂



The low selectivity for reactions of compounds (89, 90) suggests that there are limits to the ring size of substrates acceptable to SAM II. However, bicyclic structures can be hydrolysed with high enantioselectivity.^{223,225} Extremely high *E* values were obtained for the resolutions of secondary alcohols bearing a small (Me, Et) and an aromatic substituent. Lipase SAM II has also been demonstrated to give an extremely enantioselective hydrolysis of cyanohydrin acetates.²²⁹ Examples are shown in Fig. 38.

Figure 38 Cyanohydrins resolved with lipase SAM II.



The (*R*) enantiomer was hydrolysed preferentially in the cases of (93-97). For the heterocyclic compounds, (98) and (99) the selectivity was reversed and the (*S*) enantiomer was hydrolysed, but with greatly reduced selectivity.

1.5.2 The Lipase from *Mucor miehei*.

The lipase from the fungus *Mucor miehei* is available commercially either as the free enzyme or as 'Lipozyme', a preparation in which the lipase is bound to a weak ion exchange resin. Lipozyme has been designed to allow esterification reactions to be performed under vacuum (to remove water as it is formed) without losing the essential water surrounding the enzyme.²³⁰ Lipase from *M. miehei* (MML) has been found to have a very broad substrate specificity. Esterification of octanol with carboxylic acids of increasing chain length gave increasing rates up to heptanoic acid; further lengthening produced no increase in rate. Putting a methyl substituent at all positions along the chain decreased the rate and the greatest decrease was observed by branching at the 3-position. Increasing the side chain to an ethyl substituent removed activity completely unless the substituent was "tied" into a cyclohexane ring when activity was regained. Fig. 39 shows the relative rates of esterification of octanol with various acids.

Figure 39. Relative rates of esterification of octanol with various acids.

Figure 39a. Study of the effect of branching in the acid chain


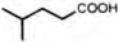
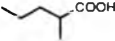
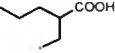
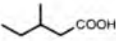



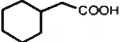
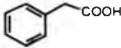

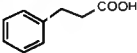
Rel. rate		Rel. rate
		
1		
		0.84
		
0.42		
		0.00
		
0.21		
		0.22

Figure 39b. Comparison of reaction rates of cyclohexyl and phenyl-substituted acids

Rel. rate		Rel. rate
		
1.41		
		
0.02		
		
4.88		
		7.95

Cyclohexyl and phenyl substituents were placed at each position along the backbone and again, inhibitory effects of branching at the beta carbon were observed. Unsaturation in the acid chain similarly reduced the rate of esterification. The esterification of 2-methylbutyric acid was used to investigate any

stereoselectivity and a slight preference for the (*S*) isomer was found. The substrate specificity for the alcohol was investigated in a similar manner and a broad range of substrates was accepted.²³⁰

The authors conclude from this information that the active site of 'Lipozyme' occupies and is surrounded by a very hydrophobic region. The selective esterification of (*R*) enantiomers of 2-octanol, 2-hexanol and 2-decanol have been reported with this enzyme.²³¹ All were esterified with an *E* value > 50 for C₆, C₈ and C₁₆ fatty acids. The selectivity for 3-octanol was reduced.

This report concluded that for high enantioselection a large alkyl group was required, unbranched in the immediate vicinity of the chiral centre.²³¹

MML has been utilised in reaction on triglycerides with advantage being taken of its 1,3 specificity on these substrates.²³²⁻²³⁶ Interesterification in supercritical carbon dioxide has also been described.²³⁵

Compounds which have been produced using MML are shown in Fig. 40.

Figure 40. Products of MML-catalysed ester hydrolysis.



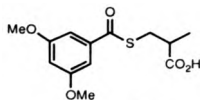
80% ee

Ref (238)



(R), optically pure

Ref (239, 240, 75)



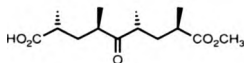
(S) 47% conv., 68% ee

Ref (241)



R = C₂H₅

Ref (243)



(-) E = 77

Ref (165)

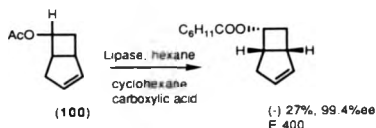


>95% ee

Ref (68)

The most striking feature of these reactions is that MML has been utilised for resolution of some secondary alcohols which have proved unsuccessful with other enzymes. An interesterification procedure has been described which improved upon the selectivity obtained with either hydrolysis or esterification.²⁴⁴

Scheme 24. Interesterification with MML.



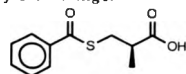
A direct esterification of the corresponding racemic alcohol with cyclohexanecarboxylic acid gave the (-) product in 48% yield and 94.3% ee ($E = 96$). The racemic acetate (**100**) was hydrolysed to the (+) alcohol (12% conv., 76% ee). The interesterification reaction gave greatly improved results as shown in Scheme 24. The authors attribute this enhanced selectivity to the fact that the substrate must visit the active site twice; once to undergo deacetylation and a second time to pick up the cyclohexanoate moiety. In fact, considering the kinetics of the reaction it simply means that the substrate is undergoing two reactions and the selectivity is naturally enhanced as the selectivity is the same for both the hydrolysis and the reesterification.

In contrast to this example, the corresponding 4,6 and 5,5 bicyclic alcohols^{75,239,73} were obtained optically pure after a single hydrolysis.

1.5.3. The lipase from *Aspergillus niger*.

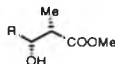
The products of reactions catalysed by lipase from *Aspergillus niger* are presented in Fig 41.

Figure 41. Products of hydrolytic reactions catalysed by the lipase from *A. niger*



(R), 32% conv., 98% ee, E 100

Ref (241, 245)



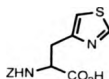
>90% ee

Ref (246)



40% conv., 60% ee

Ref (247)

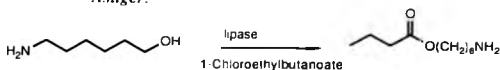


94% ee

Ref (215)

These reactions show that this lipase displays some enantioselectivity towards the acid part of a molecule. The lipase from *A. niger* has also been used for the selective acylation of either a primary amino group or a primary hydroxyl group in the same molecule ²⁴⁸

Scheme 25. Acylation of 6-amino-1-hexanol by the lipase from *A. niger*.



With the chloroethyl ester of N-acetyl-L-phenylalanine as acylating agent the amino group reacted five times faster than the hydroxyl group.

This lipase has been used in the selective removal of the 1-acetyl group from tetra-O-acetyl furanoses,²⁴⁹ and for the selective acylation of the 3-position of 6-butyryl derivatives of

glucose, galactose and mannose.¹⁷⁵ The hydrolysis of glucose pentaacetate with this lipase can be stopped at defined points to obtain tetra- and tri- acetates not available by chemical hydrolysis where removal of one acetate group accelerates removal of the next.²⁵⁰

In conclusion it may be said that this lipase has found application in some specialised areas but has not proved to have many applications in organic synthesis, as indicated by its failure in many other screening experiments.

1.5.4 The Lipase from *Pseudomonas fragi*

Most of the biotransformations reported with the thermostable alkaline lipase from *Pseudomonas fragi* have been performed with a modified enzyme. It has been modified by covalent attachment of activated polyethylene glycol (PEG),²⁵¹⁻²⁵³ and with magnetic groups.^{254,255} A lipid coated lipase compound has also been prepared.²⁵⁶

Three lipase preparations (crude, pure and PEG-modified lipases) have been compared with respect to their activity in ester synthesis from palmitic acid and cetyl alcohol.²⁵⁷

Scheme 26



The crude lipase was insoluble in all solvents and showed higher activity in water-immiscible solvents such as n-hexane, isooctane and cyclohexane. PEG lipase showed higher activity in benzene and 1,1,1-trichloroethane. Pure lipase was insoluble and virtually inactive in all solvents tested.

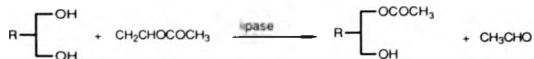
Most of the examples of the use of this lipase in the literature relate to reactions of glycerides. Elucidation of the substrate

specificity of the PEG-lipase showed that it preferred primary alcohol substrates and a longer chain fatty acid for optimum ester synthesis.²⁵⁸

Esterification of racemic and optically pure (*R*) and (*S*) secondary alcohols with dodecanoic acid showed that the (*R*)-enantiomer was esterified much more rapidly. In the case of α -phenylethanol the (*S*) isomer was a non substrate and complete enantiodiscrimination was observed.²⁵⁹

This lipase has been compared to lipase P in the reaction shown in Scheme 27.²¹⁰

Scheme 27.

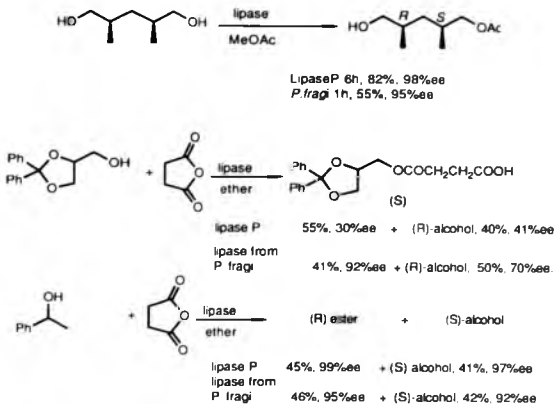


The rate of esterification with lipase from *P. fragi* was much faster but lipase P was more enantioselective. 2-Benzyl and 2-(1-naphthylmethyl)propanediols gave the *R* monoester in high chemical and optical yields with both lipases (higher ee with lipase P).

2-O-Benzyl propanetriol gave the (*S*)-monoester in the same reaction.

Further examples were found which again allow comparison of the activities of lipase P and lipase from *P. fragi*.^{210,212}

Scheme 28.

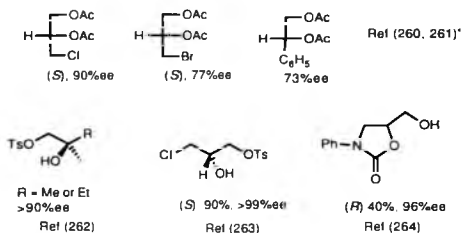


Again both enzymes showed the same preferences and in most cases lipase P showed higher selectivity.²¹²

1.5.5 The Lipase from *Pseudomonas aeruginosa*

Another *Pseudomonas* lipase has been used in a small number of highly stereoselective reactions as shown in Fig. 42.

Figure 42. Products of reactions catalysed by lipase from
P. aeruginosa



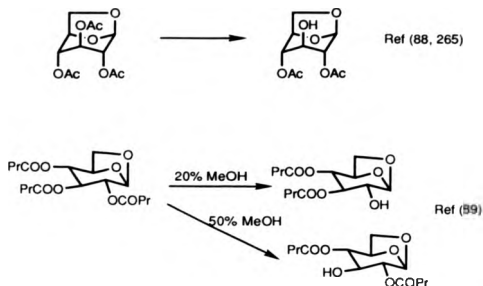
* Residual substrate from hydrolytic reactions.

The reactions discussed in references (262-264) displayed absolute stereoselectivity as was seen by the concomitant recovery of optically pure (>99%ee) starting material in all of these examples.

1.5.6. Wheatgerm lipase.

Wheatgerm lipase is the only lipase of plant origin which has been purified and used in biotransformations. Two reactions have been found in which wheatgerm lipase has been used successfully for regioselective hydrolysis of sugar esters.

Scheme 29. Regioselective hydrolyses with wheatgerm lipase.



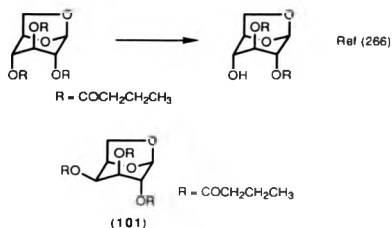
In the first example wheatgerm was the only lipase which selectively hydrolysed the C-3 acetoxy group. PPL has been reported to cleave the C-4 ester and the lipase from *Rhizopus javanicus* cleaved the ester group at C-2.

In the second example the regioselectivity moves from C-2 to C-3 with increasing methanol concentration. In these examples for chemical hydrolysis the selectivity decreased with increasing size of the acyl group but in enzymatic hydrolysis the opposite was true.

1.5.7 The lipase from *Chromobacterium viscosum*

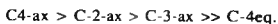
This lipase, the only one in common use from a prokaryotic organism, has been found to be useful for regioselective hydrolysis and acylation of sugars.

Figure 43. Regioselective reactions catalysed by lipase from *C. viscosum*.



In this example the stereochemistry at C-4 was critical for the reaction as the ester (101) was not hydrolysed at all.

The following order of reactivity was observed

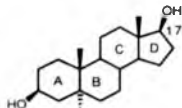


This is different from the order of reactivity for wheatgerm lipase.

The lipase from *C. viscosum* has been utilised in the selective acylation of 6-O-butyryl sugar derivatives. For example 6-O-butyryl glucose, galactose and mannose were all acylated at the 2, and 3, positions by the lipase and 2,2,2-trichloroethylbutyrate. Also, n-octyl β -D-glucopyranoside was acylated at C-6 and C-3. Again this enzyme offers different selectivity to other lipases as with CCL only C-6 was acylated and with PPL C-6 and C-2 were acylated.¹⁷⁵

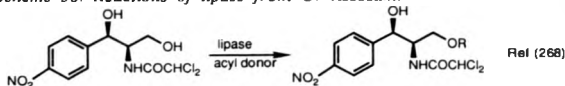
The substrate specificity of this lipase was elucidated on the esterification of a minimal steroid structure with 2,2,2-trifluoroethyl butyrate.²⁶⁶

Figure 44. Minimal steroid structure.



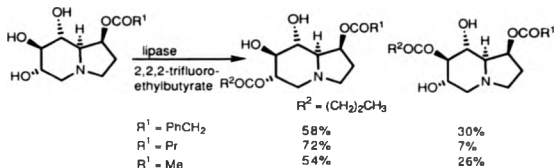
The lipase displayed absolute regioselectivity in the acylation of this and other steroids, acylating only the C-3 hydroxyl group. This was in contrast to subtilisin which displays a marked preference for the C-17 OH group and chemical acylation which did not discriminate between the two hydroxyl groups.²⁶⁶ With regard to the substrate specificity it was discovered the the substrate must have the *trans* configuration at the A-B ring junction. A double bond was tolerated in the B ring but not in the A ring and a phenolic A ring was not tolerated. Compounds with altered side chains were good substrates as long as C-3-OH and C-5H were in α - and β configurations respectively. Other reactions of this lipase are shown in Scheme 30

Scheme 30. Reactions of lipase from *C. viscosum*.

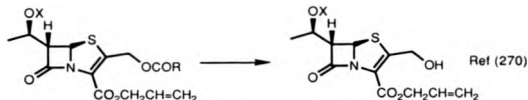


acyl donor = methyl carboxylate or trifluoroethyl ester.

(a) R = butanoyl
(b) R = cinnamoyl



Rel (269)



X = TMS (trimethylsilyl), THP (tetrahydropyranyl)

R = Pr or Me

Rel (270)

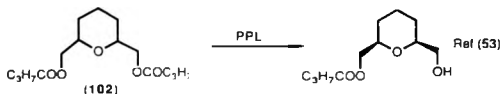
In reactions with organometallic substrates the *C. viscosum* lipase-catalysed esterification with a stannyl ether was 3 fold faster than the same reaction with the corresponding alcohol.⁶⁶ This enzyme has also been used for polymer formation from bis(2-chloroethyl)-2,5-dibromoadipate.²⁷¹

It may be concluded that this lipase may be useful for regioselective reactions of a variety of bulky cyclic structures and for some regioselective reactions on sugars and their esters. No examples have been found of this enzyme displaying stereoselectivity.

1.6 TYPICAL EXPERIMENTAL PROCEDURES FOR BIOTRANSFORMATIONS.

1.6.1. Hydrolysis with PPL

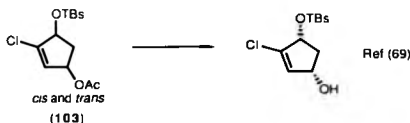
Scheme 31



The diester (102) (3.30 g, 11.5 mmol) was suspended at 25°C in buffer solution (30 ml), pH 7.0 consisting of 0.01M KH_2PO_4 , 0.1M NaCl , and 0.0025M CaCl_2 . PPL (104 mg, 12U/mg) was then added and the reaction maintained at 25°C and at pH 7.0 by pH-stat controlled titration of the liberated acid with 0.5M NaOH . After 9h, when one equivalent of base had been taken up, the mixture was filtered through Celite and extracted with diethyl ether (3 x 70ml). The combined extracts were washed with saturated aqueous NaHCO_3 , then with brine and finally dried (MgSO_4) and evaporated. The product mixture was separated by chromatography and distillation.

1.6.2. Hydrolysis with PPL

Scheme 32



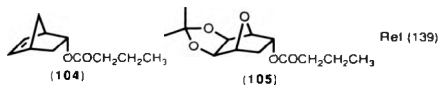
To a solution of ester (**103**) (30.0 g, 0.103 mol) in methanol (600 mL) was added 0.1M phosphate buffer (pH 7, 1800 mL) and PPL (15.0 g SIGMA L-3126). The mixture was stirred for 12h. at 15°C and then extracted with ether. The ether solution was dried (MgSO₄), and concentrated in vacuo. The residue was chromatographed over SiO₂ (450 g), to obtain acetates consisting of *trans* and *cis*-(**103**), and 6.4 g (25%) of the desired optically pure product.

Optical analysis was by HPLC of the corresponding Mosher's ester, and the product appeared to contain only one enantiomer.

1.6.3.Hydrolysis with CCL

Hydrolysis of **104** and **105**.

Scheme 33.



To a solution of lipase from *Candida cylindracea* (2.5 g) in phosphate buffer (0.1M, 250mL), pH 7.2, was added substrate ester (**104**) (20.0 g) or (**105**) (10.0 g). The mixture was stirred vigorously whilst the pH was kept constant at 7.2 by addition of 1M NaOH from an autoburette. When the appropriate degree of conversion was accomplished (40% for the optically pure product or 60% to obtain the esters optically pure) the products were extracted with dichloromethane. Evaporation of the organic solvents and subsequent column chromatography gave alcohols in 70-80% yields and esters in 80-90% yields.

1.6.4 Esterification with PPL

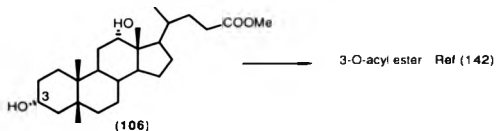
(General procedure for the esterification of secondary alcohols with dodecanoic acid).

One gram of PPL Sigma per 2.5 mmol substrate (secondary alcohol/dodecanoic acid) was used.

To a solution of each secondary alcohol (25 mmol) and dodecanoic acid (25 mmol) in n-heptane (10 ml)(twice distilled), PPL (10 g) was added and the mixture continuously stirred at defined temperatures for a defined reaction time. After cooling, the enzyme was filtered off and the volume adjusted to 50 mL using diethyl ether. This solution was analysed by GLC against a standard to measure the extent of conversion of the reaction. The heptane-diethyl ether solution was evaporated under vacuum to dryness. The residue was subjected to preparative thin layer chromatography. Quantitative yields of the pure ester and alcohol were achieved.

1.6.5 Esterification with CCL

Scheme 34.



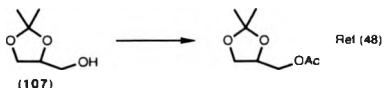
CCL (2 g) was added to 50 mL of anhydrous benzene containing 1.23mmol (500 mg) (106) and 4mmol trichloroethyl butyrate and the suspension was shaken at 250 rpm and 45°C. After

24h. a second addition of lipase (2 g) was made and the suspension was shaken for a further 24h. HPLC showed that 96% of the substrate was acylated. The enzyme was removed by filtration, the solvent evaporated and the crude residue purified by flash chromatography. The ester was isolated in 85% yield.

1.6.6 General procedure for the lipase-catalysed transesterification of alcohols with enol esters.

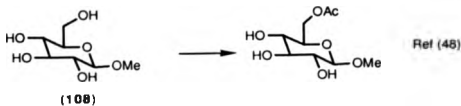
The alcohol substrate and enol ester were dissolved in a suitable solvent. The enzyme was added and the reaction monitored by GLC to determine the extent of conversion. Once the desired extent of conversion was attained the enzyme was filtered off and the solvent removed under vacuum. The ester product and unreacted alcohol were separated by chromatography.

PPL-catalysed transesterification with vinyl acetate.



Alcohol (107) (1 g, 7.5 mmol) and vinyl acetate (2.3 g, 26.7 mmol.) in 50 ml of chloroform were incubated with PPL (2 g) along with 0.5 g of toluene as an internal standard. The reaction was worked up as above after 40% or 80% conversion.

CCL-catalysed transesterification.



Methyl β -D-glucopyranoside (**108**) (388 mg, 2 mmol) and vinyl acetate (4 mmol) were dissolved in 12 ml benzene-pyridine (2:1). CCL (388 mg) was added and the suspension stirred at 28°C. After 24h. an additional batch of enzyme (388 mg) was added and this was repeated after 48h. The suspension was stirred at 28°C for 5 days and then worked up as described in the general procedure.

1.7 CONCLUDING COMMENTS

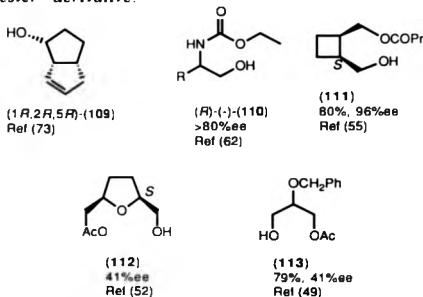
As mentioned earlier, a detailed study has not been made as to the effect of acid chain length in the enantioselectivity of hydrolysis of alcohol esters. Various studies have been reported on variation of the acid over a limited range of substrates for one or two enzymes and some of this information has been discussed in the body of the review. The aim of this section is to draw together the available data on each enzyme. Detailed information is available on the relative rates of hydrolysis of fatty acids from their respective triglycerides by various microbial lipases. Most studies concentrate on the chemical specificity of the lipases but there is evidence to suggest that physical factors also have an effect upon the specificity. For example the surface pressure affects rates of hydrolysis of substrates in monolayers by pancreatic lipase and unsaturated fatty acids are released more preferentially below 0°C than at higher temperatures in the hydrolysis of lard by various lipases.^{5b}

Comparison of the rates of hydrolysis of single triglycerides and fatty acid methyl esters with *Chromobacterium* lipase B, showed that the hydrolytic activity peaked at the C₁₀ and C_{18:2} fatty acids for triglycerides and at the C₁₆ fatty acid for fatty acid methyl esters. This selectivity was also studied with mixtures of single triglycerides using the same enzyme. C₁₄ and C₁₆ fatty acids were released preferentially from the triglycerides. The pattern for the mixtures of fatty acid methyl esters was similar to that with single fatty acid methyl esters. In the same study the substrate specificity was found to vary with temperature; specificity at 60°C was greatly different from that

at 30°C. Some reports have been found which discuss the effect of variation of the acyl chain in hydrolysis of esters of chiral alcohols.

Several of these studies were performed using PPL. In hydrolysis of esters of the following alcohols the highest selectivity was observed using the acetate ester.

Figure 45. Alcohols resolved by PPL-catalysed hydrolysis of an ester derivative.



In the cases of esters of bicyclo[3.3.0]oct-7-en-2-ol (109) an E value of 70 was calculated for the reaction with PPL on the acetate.⁷³ Extending the acyl chain from acetyl to butyryl greatly enhances the reaction rate but the enantioselectivity was virtually unchanged. This trend was also observed with hydrolysis of ester of amino alcohols (110).⁶² Little difference was observed between the reactions of acetate and butyrate diesters of alcohol (111).⁵⁵ In a similar example,⁵² the reaction was also attempted with isobutyric and pivalic esters of compound (112) but the acetate was found to give the highest selectivity. In hydrolysis of esters of alcohol (113) the best

result in terms of selectivity was again found with the acetates, as with all other esters the major product was the diol.⁴⁹ In the example shown below the highest selectivity was observed with the chloroacetate ester in hydrolysis with PPL.⁷⁷

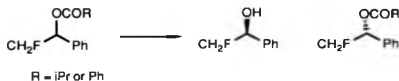
Scheme 37.



The effect of variation of the acyl donor upon the rate and selectivity of the acylation of (\pm)-menthol by CCL has been studied as discussed earlier in the review.¹³³⁻¹³⁵ The optical purity and yield of the ester were dependent upon the acyl donor. Long chain fatty acids were excellent acyl donors but gave very low selectivity. Short chain acids were poor acyl donors but showed high selectivity.

In the hydrolysis of esters of norbornane type structures variation of the acyl component showed that the acetate resulted in a slower reaction than the butyrate but no further rate increase was observed upon changing to the octanoate. The enantioselectivity was slightly reduced with the faster reacting butanoate and octanoate esters compared to the acetate. The best results in resolution of linear secondary alcohols with CCL was achieved using the isopropyl ester (33% conv., 73%ee) or phenyl ester (28% conv., 63%ee)(Scheme 33).¹⁴⁸

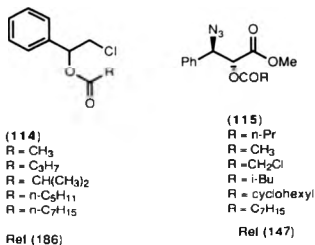
Scheme 38.



With $R =$ methyl or straight chain alkyl the hydrolysis gave products of low optical purity.

Two examples are given in Fig. 45 of variation of the acyl group in hydrolysis of esters of secondary alcohols with lipase P.

Figure 46. Esters hydrolysed by lipase P



In the case of the ester (114) variation of the acyl chain had little affect on the enantioselectivity and the product was obtained in 100%ee in each case. Reaction times increased however as the group became longer, from 20h. for $R = C_3H_7$ to 62h. for $R = nC_7H_{15}$. In the hydrolysis of ester (115) an E value >100 was obtained with $R = nPr$, Me or isobutyl, although in the latter example the reaction was very slow. The chloroacetate ester gave spontaneous hydrolysis and the cyclohexylcarboxylate group gave very slow hydrolysis. The

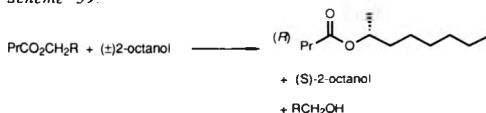
octanoate ester of (115) was hydrolysed rapidly with low selectivity.

In irreversible esterification reactions with various lipases the reactivity of enol esters was found to decrease in the order vinyl ester > isopropenyl ester > ethyl acetate > enol valerates > vinyl propionate > vinyl acetate.

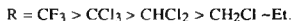
However generally the longer chain enol esters gave improved enantioselectivity.

The effect of the leaving group of the acyl donor ester on the selectivity of PPL-catalysed esterification of 2-octanol has been studied (Scheme 39)

Scheme 39.



The fastest reaction was observed with $\text{R} = \text{CF}_3$ and reaction rate decreased in the order



The most selective reaction was observed with $\text{R} = \text{CF}_3$.

In conclusion it may be said that in hydrolytic reactions of esters of chiral alcohols the acetate or butyrate ester generally proved to give the best results in terms of reaction rate and selectivity. This is true for the three lipases in most common use, namely PPL, CCL and lipase P. Increasing the acyl chain length beyond butyrate seems to give faster, less selective reactions.

In acylation reactions long chain acyl donors generally offer faster reactions. With CCL, however short chain fatty acids offer

greater selectivity. In the cases of enol esters as acylation agents longer chain esters give higher selectivity with all of the enzymes.

A survey of the number of reactions catalysed by each enzyme on each particular substrate type is summarised in the table below.

Table 8. Number of esterolytic reactions on each substrate type catalysed by each of the common enzymes.

Enzyme (source)	primary alcohol	secondary alcohol	acid
PPL	32	32	10
CCL	8	42	18
lipase P	15	36	9
SAM II	2	4	0
<i>M. miehei</i>	4	11	2
<i>A. niger</i>	2	4	3
<i>P. fragi</i>	5	3	-
<i>P. aeruginosa</i>	1	3	-
<i>C. viscosum</i>	2	4	-

From these figures and the foregoing discussion it is immediately apparent that PPL, CCL and lipase P are the most frequently used enzymes. It is unclear whether this is due to the fact that other enzymes are not screened for the desired reactions or whether they are simply the most selective enzymes. It is interesting to note the change in fashion in the use of enzymes; most of the PPL-catalysed reactions have been reported during 1986-1988. In contrast the *Pseudomonas* lipases have been more recently established with the majority of their applications reported in 1988-1990. However, despite this PPL is the obvious choice for reactions involving a primary alcohol. CCL has proved to have little application to these

compounds and although lipase P has been successfully applied in some cases these are greatly outnumbered by the number of secondary alcohols successfully resolved.

In the resolution of secondary alcohols the enzymes of choice are CCL and lipase P or lipase SAM II. Although the latter has only been applied in a limited number of cases it displays almost complete enantiodiscrimination.

In hydrolysis of the following generalised substrates for esters of linear and cyclic secondary alcohols (Fig. 47) PPL exhibits an overwhelming preference for structure type (a) but a more relaxed selectivity toward the cyclic structures.

Figure 47. Generalised substrate esters for hydrolysis.

A : linear secondary alcohols

B : cyclic secondary alcohols



(a)



(b)



(c)



(d)

where S = small alkyl and L = large alkyl

CCL shows variable selectivity towards both structure types shown in Fig. 47, although a bias is shown towards type (a) of the linear structures and type (d) of the cyclic ones.

Although in certain cases discussed earlier, PPL and lipase P have displayed similar selectivities toward primary alcohols, in the case of linear secondary alcohols lipase P is strongly biased toward structure type (b). In the instances of hydrolysis of esters of cyclic compounds lipase P is selective predominantly for structures of type (d).

For reactions on sugars and nucleosides PPL is preferred over lipase P and CCL. Here though, one of the less popular lipases

such as that from wheatgerm or *Chromobacterium viscosum* may be applicable.

The size of the active sites of these enzymes seems to vary. For instance there are reported cases of CCL and lipase from *C. viscosum* being active on steroids whereas none of the other enzymes, apparently has got this capacity.

As would be expected, considering their true substrates, lipases are less frequently used for reactions on chiral acids. CCL has however found the greatest number of applications in this area. Many other lipases, particularly from microbial sources, have been used for a very small number of application. These have not been discussed here because insufficient data are available to deduce whether there are any specific trends in their reactions.

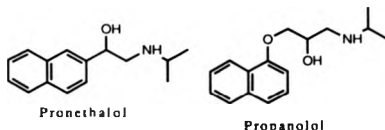
CHAPTER 2

RESOLUTION OF A BETA BLOCKER PRECURSOR

2.1 INTRODUCTION

Beta-blockers - compounds which selectively block β_1 cardiac receptors - have found many clinical uses. Stimulation of the β -cardiac receptors increases rate and force of contraction of the heart. Selective blocking of these receptors gives relief from angina, certain kinds of cardiac arrhythmia and high blood pressure. Pronethalol was the first clinically available beta blocking agent but was withdrawn from the market because of its toxic side effects in animals.²⁷²

Figure 48. Structures of early beta blockers.



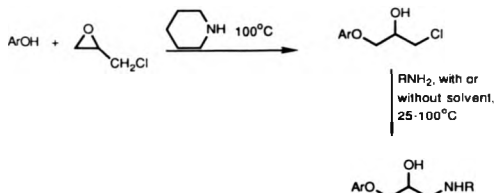
Further work to optimise the β -antagonist activity led to modification of the aryl ring and amine substituent as well as the introduction of linking groups between the aryl ring and the ethanolamine chain. Oxymethylene proved to be the best linking group as seen with propanolol which is 10-20 times more active than pronethalol.

Since the start of the development in the early sixties many successful drugs have been marketed. Propanolol is the reference compound for assessment of activity. The

β -antagonist activity of these compounds resides in one enantiomer - the (*S*)-aryloxypropanolamine. However most β -blockers are sold commercially as racemates. Normal resolution techniques involving optically active acids were time-consuming and unreliable and much effort has been devoted to devising an asymmetric synthetic route which would provide both enantiomers reliably.

The normal route to β -blockers involves base-promoted reaction of the corresponding phenol with epichlorohydrin which can give either the corresponding epoxide or α -chlorohydrin, which are then reacted with the appropriate amine.

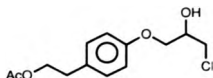
Figure 49. General β -blocker synthesis



The less active (*R*)-enantiomer of one such compound has been synthesised starting from D-mannitol but this is not an attractive route to the (*S*)-enantiomer because L-mannitol is not readily available. The (*S*)-enantiomer is available through a reaction starting from glycerol acetonide, and an elegant route has been devised to optically active epichlorohydrin from which the (*S*)- β blocker is prepared.²⁷²

The aim of the investigation described in this chapter was to provide a simple enzyme-catalysed resolution of the racemic chlorohydrin precursor to the β -blocker family. If this compound were available in optically pure form, reaction with an appropriate amine would generate the biologically active enantiomer of the β -blocker. Accordingly resolution of the following compound was attempted *via* lipase-catalysed hydrolysis of the corresponding butyrox ester.

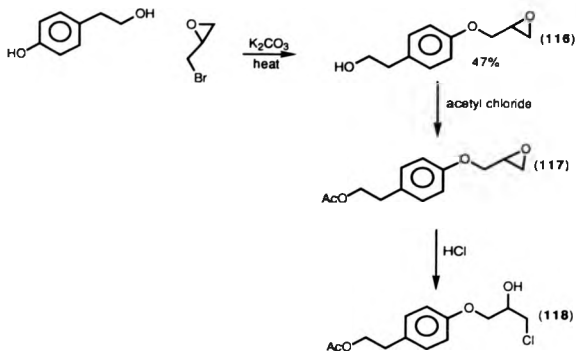
Figure 50. The β -blocker precursor to be resolved.



2.2 SYNTHESIS OF THE RACEMIC α -CHLOROHYDRIN

The racemic α -chlorohydrin (118) was synthesised by the following route.

Scheme 40. Synthesis of the racemic compound.

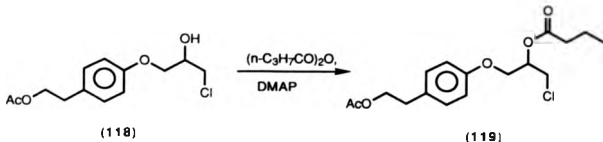


3-[4-[2-Hydroxyethyl]phenoxy]-1,2-epoxypropane (116) was prepared by reaction of 4-hydroxyphenethyl alcohol and epibromohydrin in butanone with potassium carbonate as the base in a heterogenous reaction mixture. Treatment of epoxide (116) with triethylamine and acetylchloride in dry THF gave the corresponding acetate (117). The epoxide ring was then opened by treatment with dry HCl gas in dichloromethane to give the desired 1-chloro-2-hydroxy-3-[4-[2-acetoxyethyl]phenoxy]propane (118). The methine proton in the desired isomer would be expected to give a signal at δ_H 3.4-4.1 ppm in the 1H NMR spectrum. The corresponding proton in the unwanted isomer would typically be expected to resonate at δ_H 3-3.5 ppm. The actual resonance is observed at

δ_{H} 4.2 ppm suggesting that the secondary alcohol has formed, as would be expected from this reaction.

The corresponding butyrate was prepared by treatment of the α -chlorohydrin (118) with butyric anhydride in pyridine in the presence of a catalytic amount of *N,N*-dimethylaminopyridine.

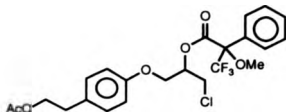
Scheme 41. Esterification of the α -chlorohydrin.



2.3 A METHOD OF CHIRAL ANALYSIS

For effective analysis of optically active products from enzymatic hydrolysis of the butyrate ester, the Mosher's ester of the α -chlorohydrin was prepared. Previous experiments on this system²⁷³ had shown that signals attributable to the methyl groups in the diastereoisomeric derivatives gave baseline splitting in the ^1H NMR spectrum. This was confirmed by preparation of the Mosher's ester of the racemic α -chlorohydrin and this method was used in chiral analysis of samples from the enzymatic hydrolyses.

Figure 51. Mosher's ester of the α -chlorohydrin.



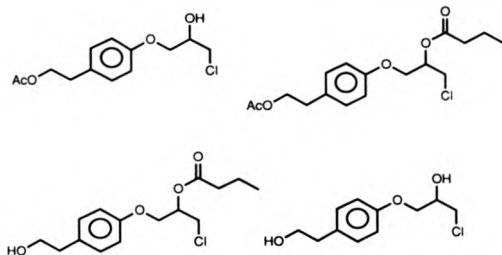
2.4 ENZYMATIC HYDROLYSES OF DIESTER (119)

Screening experiments on the hydrolysis of diester (119) with various lipases had been performed earlier.²⁷³ It had been established that lipases from *Mucor* and *Rhizopus* sp. (ex Amano) would hydrolyse the ester. The reactions appeared to stop at 50% completion and gave enantiomerically pure α -chlorohydrin (118) on the basis of ^1H NMR at 270MHz of the Mosher's ester. These experiments were, however, performed only on an analytical scale (20 mg substrate). The aim of this section of the investigations was to scale-up the experiments and to isolate the enantiomerically pure β -blocker precursor in greater quantity.

Reactions were performed on 500 mg of the racemic substrate with both lipases. The hydrolyses were performed on a pH stat to maintain constant pH by continuous addition of sodium hydroxide solution. The reactions were carried out in phosphate buffer with and without a 10% acetone cosolvent and were worked up by extracting with ether, followed by drying and evaporating. The α -chlorohydrin was isolated with 80-95%ee from experiments with both enzymes. It should be noted that the same isomer was hydrolysed by both enzymes,

although no attempt was made to assign the absolute configuration as this work was being performed elsewhere on a very closely related compound.²⁷³ The recovered product showed that the enzymes were mainly specific for the butyryloxy moiety, although some desacetoxy product and a trace of diol were also observed on TLC.

Figure 52. Products recovered from the enzymatic hydrolysis.



The reactions were observed to be extremely slow, taking several days to reach the desired conversion (namely uptake of 50% of the theoretical amount of alkali from the autoburette). This meant that absorption of carbon dioxide from the atmosphere was rendering the autotitrator traces unreliable. Instead of giving the anticipated smooth curves the alkali was apparently added in a series of bursts. In some instances the reactions appeared to stop after approximately 30% completion. A control without enzyme showed little uptake of alkali. Furthermore when the reactions were worked up, the relative proportions of product and remaining substrate suggested that

the hydrolysis was in fact only 10-20% complete. However the total recovery was also very poor which could mean that the product was simply not being extracted.

To avoid this problem the work up procedure was changed. The aqueous phase was evaporated from the reaction mixture to leave a mixture of organic materials, enzyme and buffer salts. This was then extracted with dry ethyl acetate and the organic extract was filtered and evaporated. This procedure improved the recovery but the ratio of recovered starting material to product was unchanged. To check whether solubility could be causing problems a further reaction was performed using lipase from *Mucor* sp. and a solvent system comprising a 1:1 mixture of isooctane: buffer. The reaction was again monitored by pH stat. The reaction stopped abruptly when it was 6% complete. There appeared to be no problem regarding evaporation of the isooctane.

At this point it was decided to develop an HPLC system to monitor the composition of the reaction mixture more closely as the reaction progressed. Attempts were made to dissolve authentic product and the ester in mixtures of methanol and water. 70% Methanol was required to dissolve both compounds and so this was the solvent system used with a C-18 reverse phase column. The retention times for the alcohol and the ester were 8 min and 38 min respectively from the solvent peak, and so this system was used to follow the reactions. The reactions were simply stirred at room temperature using a 1M phosphate buffer solution and a 10% acetone cosolvent. Samples were removed at intervals and evaporated to dryness. The residue was taken up in ethyl acetate and this extract was dried and

evaporated. The residue from the organic extract was then dissolved in methanol for HPLC analysis.

This method of monitoring the reaction showed that the hydrolysis with both lipases proceeded rapidly until the mixture contained a certain amount (~8.5%) of the α -chlorohydrin product after which no further hydrolysis was observed over several days.

It was suspected that the enzymes were of low activity or that they were becoming denatured by prolonged exposure to water and the atmosphere.

The activities of the two enzymes were checked by hydrolysing tributyrin with each of them in reactions monitored by the autotitrator. A steady uptake of alkali was observed in each case as the ester was hydrolysed, showing that the enzymes were displaying activity. A reaction mixture with each enzyme was then prepared as previously and allowed to stir at room temperature for 24 h. These mixtures were equilibrated on the autotitrator and tributyrin was added. The ensuing alkali consumption showed that no enzyme activity had been lost. Another possible explanation as to why the enzymes are not particularly active on this substrate is that they are becoming inhibited by the product α -chlorohydrin once it reaches a certain concentration. Reaction mixtures were prepared which contained substrate (20 mg) and 10-20 mol% of the racemic α -chlorohydrin. The reactions were again followed by HPLC using the procedure described earlier. This experiment was repeated several times and in each case some doubt was cast upon the validity of the HPLC traces because of problems with reproducibility. In general the proportion of the α -

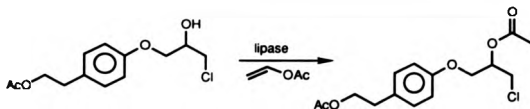
chlorohydrin was observed to increase to approximately 27% over 24h. If the reactions were monitored over a period of 7-10 days, the appearance of impurities was observed on the chromatogram. These were not identified. Hence, although the possibility cannot be ruled out that just one enantiomer of the product inhibits the reaction it really appears that this is not the explanation.

Following this result, the more likely possibility that the butyric acid byproduct of the reaction was inhibiting the enzyme was investigated. Reaction mixtures were prepared as previously but 10-20 mol% sodium butyrate was added to the mixture. In this case reactions were followed by TLC as the alcohol spot should appear as the reaction proceeds. Over the course of a week traces of one other spot appeared near the baseline on TLC but nothing corresponding to the anticipated product was observed. Hence it seems that these particular enzymes are inhibited by butyric acid in the reaction mixture.

2.4 TRANSESTERIFICATION OF α -CHLOROHYDRIN (118)

In an attempt to avoid the enzyme inhibition a transesterification reaction was performed with both enzymes using vinyl acetate.

Scheme 42. Transesterification of the α -chlorohydrin.



The appropriate enzyme (10-20 mg) was simply stirred with 20 mg substrate in 0.5 ml vinyl acetate at room temperature. Reaction was followed by TLC, eluting with ethyl acetate: petrol 1:1 which gave an R_f value of 0.3 for the substrate. Removal of the polar hydroxyl group by esterification should mean that the product will have a higher R_f value. The reactions were monitored for 1 week and even after this time no product was evident in either of the reactions.

2.5 SUMMARY AND CONCLUSIONS

The racemic β -blocker precursor was prepared and esterified successfully, although yields were not high.

In the enzymatic hydrolysis the optically active product was obtained in high enantiomeric excess but in very low yield. Further work showed that this appeared to be due to enzyme inhibition by either the enantiomer of the product formed or (more likely) by the butyric acid formed as byproduct. It is possible that a system could be devised whereby a solution of the substrate is passed slowly and continuously over a column of immobilised enzyme. This should avoid the problem of butyric acid inhibiting the enzyme.

The second possibility, namely that of using an esterification reaction to avoid inhibition was totally unsuccessful as no reaction was observed with either enzyme.

CHAPTER 3

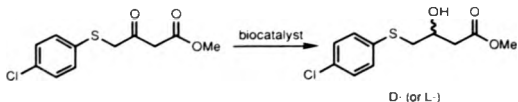
RESOLUTION OF METHYL 4-(p-CHLOROPHENYLTHIO)-3-HYDROXYBUTANOATE.

3.1 INTRODUCTION

The aim of this investigation was the resolution of another secondary alcohol, again via lipase catalysed hydrolysis of an ester.

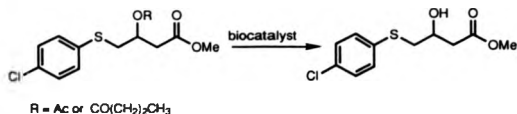
Methyl 4-(p-chlorophenylthio)-3-hydroxybutanoate had already been obtained in high optical purity via stereospecific microbiological reduction of the corresponding keto-ester.²⁷⁴

Scheme 43 Reduction of methyl 4-(p-chlorophenylthio) 3-oxobutanoate



In this reduction either enantiomer could be obtained in excess by careful choice of biocatalyst. The L-enantiomer was obtained in 40% yield and 50%ee by reduction with *Saccharomyces cerevisiae* and the D- enantiomer in 30-38% yield and 80%ee by strains of *Candida guilliermondii*. Screening experiments had also been performed on the lipase-catalysed hydrolysis of the corresponding acetate and butanoate esters.

Scheme 44. Resolution by hydrolysis.



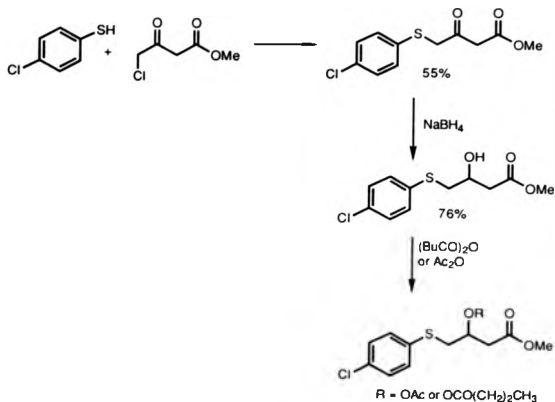
In the work described in this chapter both esters were prepared and resolved by hydrolysis with PPL (for the acetate) and with lipase P (for the butanoate).

3.2 SYNTHESIS OF THE RACEMIC ESTERS.

Esters were prepared by condensation of *p*-chlorothiophenol and methyl 4-chloroacetate, followed by reduction and esterification (Scheme 45).

The condensation reaction was performed in pyridine and the yield quoted was for a small scale experiment. The product was purified by recrystallisation and was identified unambiguously from its ^1H NMR spectrum. The reduction step proceeded smoothly in THF at -20°C , and again the product was purified by recrystallisation. The acetate was prepared by stirring with acetic anhydride in pyridine at room temperature. The butanoate was prepared by a similar procedure, but DMAP was added as a catalyst. Yields for the acetate and butanoate were 40% and 62% respectively. Both were purified by flash chromatography and their identities were confirmed by NMR.

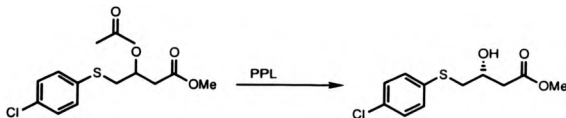
Scheme 45. Ester synthesis.



3.3 HYDROLYSIS OF METHYL 3-ACETOXY-4-(p-CHLOROPHENYLTHIO)BUTANOATE.

Hydrolysis of the acetate was performed in aqueous systems and followed by consumption of base from a pH Stat.

Scheme 46. Hydrolysis of the acetate.



The product and unreacted starting material were extracted and separated by preparative TLC or by flash chromatography. Enantiomeric excess was determined by ^1H NMR at 220 MHz using approximately 5 mol% of europium D-3-trifluoroacetylcamphorate in deuteriochloroform. This system had earlier been shown to give good splitting of the methoxy signal as long as the compound, shift reagent and solvent were anhydrous. If not, line broadening was observed. Under optimal conditions the signal attributable to the minor component could not be detected when the compound had an $ee > 95\%$.²⁷⁴

The first hydrolytic experiments used a low concentration of the substrate in phosphate buffer and a very slow conversion was observed. Working up a reaction which was stopped at 34% conversion (as judged by alkali consumption) gave racemic alcohol and recovered starting material with a specific rotation of $[\alpha]_D +2.7^\circ$.

When the concentration of the substrate in the phosphate buffer was increased (to improve the emulsifying properties of the mixture) no hydrolysis was observed. Also, use of a highly purified PPL (available from Sigma) gave no hydrolysis in the initial system. To improve reaction rate and to produce a smooth reaction curve the hydrolyses were performed with a cosolvent. Addition of 10% acetone improved the reaction rate and produced a smoother curve of alkali addition. Addition of 10% methanol produced a smooth reaction curve and a definite rate change was observed at 50% conversion. This was not quite reproducible as sometimes the addition of alkali would stop for several hours for no apparent reason and then continue

as previously. This reaction was repeated on a larger scale and was stopped at a conversion of 38%. Isolation and analysis of the product showed that it was formed in 64%ee. Christen had previously related the signals in the spectrum of the product determined in the presence of the shift reagent to the absolute configurations of the enantiomers. On the basis of this work it was seen that the (*S*)-enantiomer of the product had been formed by the PPL-catalysed hydrolysis. The configuration and enantiomeric excess were confirmed by measurement of the optical rotation. The rotation of the pure (*S*) enantiomer is reported to be -5° and in this case a rotation of $[\alpha]_D -3.11^{\circ}$ was obtained.

3.4 HYDROLYSIS OF METHYL 3-BUTANOYLOXY-4-(p-CHLOROPHENYLTHIO)BUTANOATE

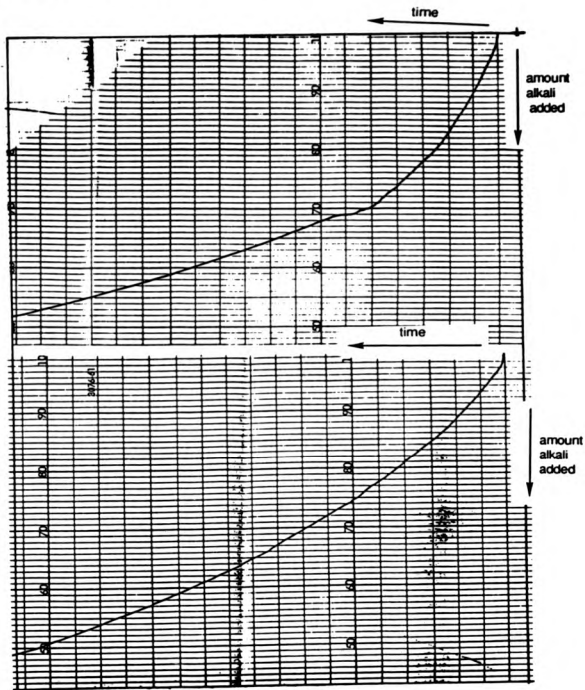
In an attempt to improve upon the enantioselectivity obtained in the hydrolysis of the acetate the corresponding butanoate ester was prepared and hydrolysed in a similar manner. On the basis of screening experiments performed elsewhere²⁷⁴ lipaseP from *Pseudomonas fluorescens* was used for the hydrolysis of methyl 3-butanoyloxy-4-(*p*-chlorophenylthio)butanoate. This enzyme is selective for the opposite enantiomer and so provides a convenient route to the other isomer.

Scheme 47. Hydrolysis of the butanoate ester.



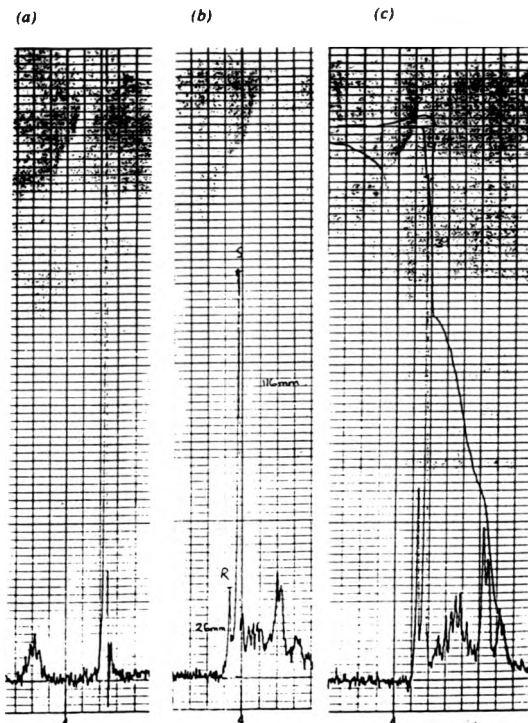
The hydrolysis was performed on a larger scale on the pH-stat in phosphate buffer with a 15% methanol cosolvent, with and without the addition of a small amount of Triton X-100 to aid emulsification. In parallel experiments with and without the surfactant it was unclear whether this was actually of any benefit in the reaction. In general the reactions with surfactant were faster and sometimes gave a clear apparent rate change at approximately 50% conversion (autotitrator traces are presented in Fig. 53). Preparative scale work was performed with surfactant because the reactions were faster. The disadvantage of this system is that on some occasions dense, stable emulsions formed on extraction which were difficult to disperse even on filtration through Celite.

Figure 53. Autotitrator traces for the hydrolysis with and without Triton X-100.



The reaction was performed on a preparative scale (500 mg substrate) and the product and remaining starting material were isolated by flash chromatography after extraction. The enantiomeric excess of the product was determined by ^1H NMR at 220 MHz as described previously. In this instance the *R*-enantiomer of the product alcohol was isolated in 72% yield (of the theoretical 50%) with 88%ee. Addition of a small amount of authentic racemic material increased the size of the peak due to the minor enantiomer relative to that of the major isomer, demonstrating that the system was giving a true indication of the optical purity (see Fig. 54).

Figure 54. Methoxy peaks of the ^1H NMR spectra of the (*S*) enantiomer of the optically enriched alcohol, (a) alone (b) + chiral shift reagent, (c) + chiral shift reagent + authentic racemic alcohol.

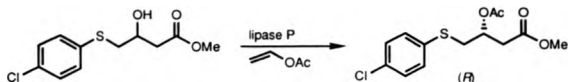


The rotations of the product and recovered starting material were $[\alpha]_D +5.36^\circ$ (1.12% CHCl_3) and $[\alpha]_D +9.72^\circ$ (1.96% CHCl_3) respectively. One recrystallisation from dichloromethane and pentane raised the enantiomeric excess of the product from 87% ee to >95%. It proved impossible to determine the enantiomeric excess of the recovered ester directly using an NMR method with a lanthanide shift reagent. Attempts were therefore made to hydrolyse the ester using sodium methoxide, following a standard procedure.²⁷⁵ Experiments using the racemic ester however, showed that the desired reaction was not occurring. The ester was finally hydrolysed using an enzyme cocktail of PPL (to hydrolyse the main (*S*) enantiomer) and a small amount of lipase P (to hydrolyse the (*R*) minor enantiomer). These reactions took approximately 24h. to reach completion. The (*S*) isomer of the alcohol was obtained in 40-50% ee (again determined using NMR with the chiral shift reagent). Calculation of the exact degree of conversion and of the E value (enantiomer ratio as described in the introduction) for two separate reactions shows that the reaction has an E value of 18-27. Hence this reaction can be useful on a preparative scale especially as the optical purity of the alcohol is readily increased by recrystallisation.

3.5 TRANSESTERIFICATION REACTIONS.

As a complementary route to the different enantiomers of the alcohol an irreversible transesterification was performed with vinyl acetate.

Scheme 48. Transesterification reaction.



The (*R*) ester will be formed by this reaction with lipase P because the enzyme will work upon the same enantiomer as in hydrolysis. Thus the (*S*) enantiomer of the alcohol can be recovered from the reaction mixture.

The reaction was performed in *tert*-butyl methyl ether using three equivalents of vinyl acetate. These conditions had been established previously, in an investigation of rates of transesterification with increasing amount of vinyl acetate.²⁷⁶ This reaction was followed by TLC and worked up when it was approximately 50% complete. The enzyme was removed by filtration through a glass sinter, the solvent was evaporated and the compounds separated by flash chromatography. The recovered alcohol (*S*- enantiomer) was isolated in 62.5%ee. The newly formed ester was isolated and hydrolysed using PPL and lipase P as described earlier for the butanoyloxy ester. Analysis by NMR showed that the ester had been formed in 53% enantiomeric excess.

This gives an E value of 6 for the transesterification. A further transesterification reaction was terminated at 32% conversion (calculated from the optical purities of substrate and product) and gave the remaining substrate in 36%ee and product ester in 75%ee. This represents an E value of 10. The optical purity of the alcohol was increased to >95%ee by two recrystallisations from dichloromethane:pentane, although the final yield of the optically pure alcohol was low.

3.6 CONCLUSIONS.

Methyl 3-hydroxy-4-(*p*-chlorophenylthio)-butanoate was prepared and resolved *via* hydrolysis of its corresponding esters and by a transesterification reaction in organic solvent. The E values for the hydrolysis reaction show that the optically pure alcohol or ester may be obtained on a preparative scale. The transesterification reaction has a lower E value but is still useful as a simple method of production of the opposite enantiomers of ester and alcohol. The ee of either enantiomer of the alcohol obtained can be readily increased by recrystallisation from dichloromethane and pentane.

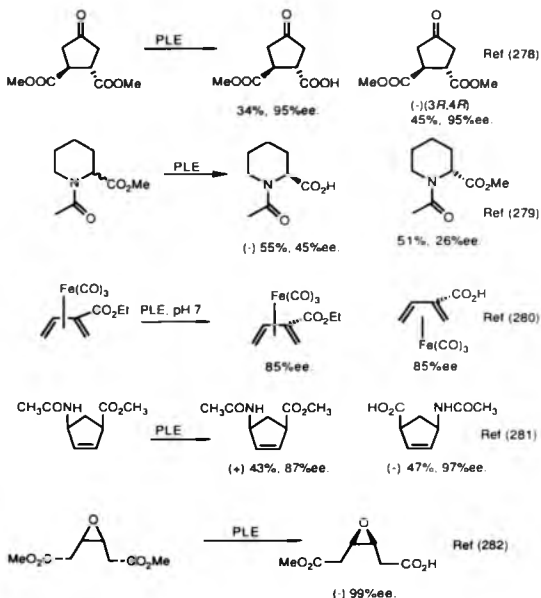
CHAPTER 4
RESOLUTION OF 3-METHYL-4-OXO-4-(4-AMINOBENZYL) BUTANOIC
ACID

4.1 INTRODUCTION

The investigations described in chapters 2 and 3 were directed towards the resolution of chiral secondary alcohols. In this section the aim of the work described was to resolve a chiral acid, again by enantioselective hydrolysis of an ester of the racemate. Lipases have been used in the work on chiral alcohols because they are most suited to this owing to the nature of their natural substrates. In this instance although some lipase-catalysed reactions were attempted, the resolution was largely investigated using an esterase. Esterases are another class of hydrolytic enzymes which have been used frequently in biotransformations. Compared to the lipases there are relatively few commercially available esterases and the majority of the reactions of this class of enzyme have been performed with pig liver esterase (PLE). This enzyme has been demonstrated to consist of six major isoenzymes, all of which are trimers of three individual proteins and all of which have substantially equal stereoselectivities.²⁷⁷ The natural function of PLE in the liver is to hydrolyse various esters in the porcine diet and it has been found to have a broad substrate specificity. PLE has been most widely applied for hydrolysis of *meso* compounds to give high yields of optically active products. However, in the instances where it has been applied to kinetic resolutions it is generally for the resolutions of esters of chiral

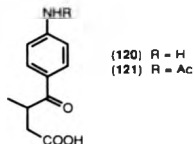
acids by a hydrolytic procedure. Some examples of acids resolved using PLE-catalysed hydrolysis of an ester are shown in Fig. 55.

Figure 55. Examples of PLE-catalysed resolutions



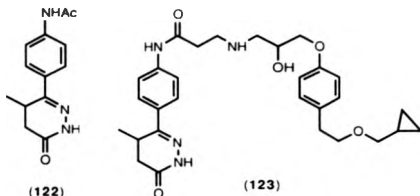
The compound which it was wished to resolve in this project is shown in Fig. 56.

Figure 56. The compound to be resolved



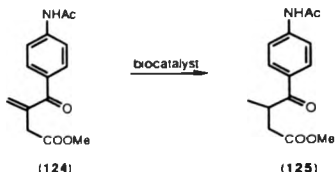
This particular acid was a precursor to a potent vasodilator (122)(Fig. 57). A simple method was sought for the preparation of the enantiomers of compound (122) for preparation of all possible stereoisomers of a novel antihypertensive agent (123) with combined vasodilator and β -adrenoceptor antagonist activity.

Figure 57. The vasodilator(122) and the target drug molecule (123).



The most obvious method of resolution which was applied was PLE-catalysed hydrolysis of an ester and this was investigated thoroughly. The second approach involves microbial reduction of the corresponding unsaturated compound as shown in Scheme 49

Scheme 49 Resolution via microbial reduction



There are a great many reported biotransformations which have been performed with bakers' yeast. A review published recently covers the subject of bakers' yeast catalysed reactions in detail.²⁸³ Microbial transformations of carbon-carbon double bonds have been mainly applied in the synthesis of intermediates to the chiral isoprenoid unit found in natural products such as tocopherol, phytol and other insect pheromones. The substrates accepted for these reactions cover mainly "activated" carbon-carbon double bonds with a wide variety of functional groups attached, in the main, to a triple substituted double bond as in Fig. 58.

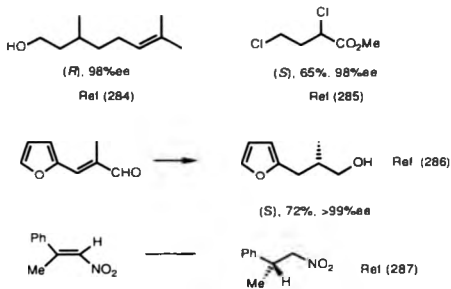
Figure 58. General formula of unsaturated compounds reduced by baker's yeast.



R¹ = alkyl, CH₂OH, CO₂R, NO₂, CHCl₂, vinyl, PhCH=CH-aryl, 2-furylthienyl, 2-(1,3-dithiacyclohexyl), phenyl sulphonyl, phenylthio-
 R² = CH₃, CF₃, Cl, Br, F.
 R³ = alkyl, CH(OR)₂, CHO, CH₂OH, CO₂R, Ph, COR

Fig. 59 shows compounds obtained obtained via bakers' yeast catalysed reduction of a double bond.

Figure 59. Compounds obtained by reduction of a double bond using bakers yeast.

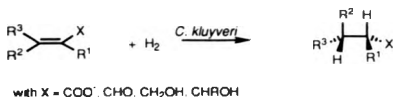


In the case of compound (124) the reduction was attempted using bakers' yeast, a strain of the yeast *Candida guilliermondii* and several strains of *Clostridia*, a *Lactobacillus* strain and *Klebsiella aerogenes*

The anaerobic spore forming bacteria of the genus *Clostridia* were discovered by Pasteur in the mid nineteenth century. Many strains of *Clostridia* are characterised by a fermentation of soluble carbohydrate to give acetic and butyric acids, CO₂ and H₂. Such bacteria will not grow in a complex medium in the absence of a source of fermentable sugar. One remarkable strain, *Clostridium kluyveri* which was used for the reduction grows only at the expense of ethanol and acetate as an energy source. These are fermented to the fatty acids butanoate and caproate.

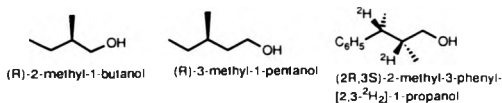
Clostridia produce a variety of exotoxins which are disease causative agents in animals and humans. They are also responsible for the anaerobic decomposition of protein in putrefaction.²⁸⁸ As yet however there are few examples of the use of these bacteria in biotransformations. Almost all of the work carried out to date has emerged from the group of H. Simon in Munich. Much of this work has concentrated on the hydrogenations of the following type using resting cells.

Scheme 50. Reduction with Clostridia sp.



This reduction with *C. kluyveri* was used to give the following optically pure products.²⁸⁹

Figure 60. Examples of optically pure compounds obtained by reduction with C. kluyveri.



The reduction of allyl alcohols has also been studied using resting cells as well as crude extracts of two species of *Clostridia*.²⁹⁰ Stereospecific reductions have also been studied with the free and immobilised cells and isolated enzymes.²⁹¹ It was found that the whole cells (free or immobilised) were more stable than either free

or immobilised isolated enzymes. The reduction of E-2-butenate was studied in these experiments.

2,3-Unsaturated acids have been reduced with *C. kluyveri*.²⁹²

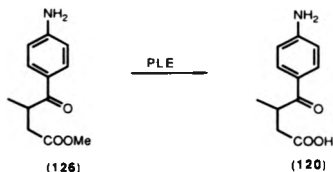
Most of the studies have been approached from a biochemist's viewpoint, with the mechanism and possible use of various enzyme systems being elucidated for these hydrogenation reactions. However these reports serve to demonstrate that *Clostridia* strains are capable of stereospecific hydrogenation reactions and so could be useful in the reduction of compound (124) to give optically active compound (125).

The attempts to produce compounds (120) and (121) in optically pure form will now be considered in detail.

4.2 HYDROLYSIS WITH PLE

The resolution of 3-methyl-4-oxo-4-(4-aminobenzyl)butanoic acid was attempted via enantioselective hydrolysis of its methyl ester as shown in Scheme 51.

Scheme 51. The hydrolytic reaction attempted



A variety of cosolvents were used since the ester proved to be completely insoluble in water. Reactions were performed using a titrating pH stat unit so that the pH of the mixture could be maintained at a constant pH and that the extent of hydrolysis could be determined by the amount of alkali consumed. Use of either 10% acetone or 10% methanol as a cosolvent however, led to some uptake of alkali in control experiments performed without addition of enzyme. Extraction of the starting material and 'product' from these control experiments showed that no hydrolysis had actually occurred, as the substrate was recovered unchanged. This means that in the enzyme-catalysed hydrolyses using these cosolvents the degree of conversion cannot be estimated from the autotitrator trace. The hydrolyses were performed with PLE in thermostatted vessels at 35°C and were stopped at varying extents of conversion. The product acid was extracted at pH 2 and the remaining substrate was extracted at pH 8.

The reactions performed with a 10% acetone cosolvent produced poor reaction curves from their alkali consumption and when worked up showed that little hydrolysis was occurring. With a methanol cosolvent some hydrolysis was found to occur and the product was isolated. Neither product nor recovered substrate showed any optical rotation however, showing that the reaction was non-stereoselective.

It has been demonstrated previously that the enantioselectivity of PLE-catalysed hydrolytic reactions is affected by the amount and nature of the cosolvent used. DMSO has been shown to be a good

cosolvent²⁹³ and in two studies variation of the amount of DMSO added showed that maximum optical purities were obtained with addition of 25-50% of this cosolvent.^{294,295} Consequently the hydrolysis of compound (126) was attempted, again with PLE in a system comprising 0.2M Tris buffer with a 25% DMSO cosolvent. In this case a control experiment with no enzyme performed on the autotitrator showed no consumption of alkali over 24 hours. A small scale experiment with PLE showed a smooth uptake of alkali with a plateau when 50% of the theoretical amount of the sodium hydroxide had been consumed. This suggested that the enzyme was hydrolysing the two enantiomers at different rates. This reaction was scaled up to isolate the product and remaining substrate at 50% hydrolysis and to investigate the optical purity of these compounds. Problems were encountered with the work-up procedure from this solvent system as the DMSO coextracted with both the product and remaining starting material and the Tris buffer salts also extracted with the acid. When rotations of the crude products extracted were measured it was found that ester had a rotation of $[\alpha]_D +3.45^\circ$. Various columns and solvent systems were tried with the crude mixture to "filter out" the contaminants but no success was achieved using this approach. Failing this, a countercurrent extraction procedure was devised. The crude ester was dissolved in dichloromethane in the first of a series of 10 tubes which each contained an equivalent amount of this solvent. The ester fraction was washed with water. The water was separated and passed into the second tube and fresh water was added to the first tube. Both were shaken and the water layers

separated and moved one tube further down the line. Fresh water was again added to the original tube. This process was repeated until all of the tubes contained two layers. NMR analysis of the dichloromethane layers showed that the ester was located in decreasing amounts in tubes 1 to 4. DMSO was located in the aqueous phase of tubes 9 and 10.

The acid was treated in a similar manner. It was dissolved in ethyl acetate and transferred along a line of 10 tubes, the first two containing pH2 buffer and the remainder containing water. Fresh ethyl acetate was added to the first tube at each step.

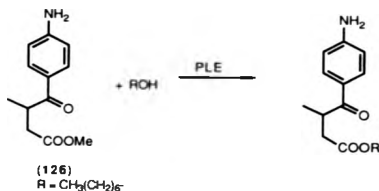
Further experiments were performed using the aqueous DMSO solvent system and the products were purified using the counter current technique. The initial products from the extraction/purification procedure exhibited some optical activity, but after further purification by crystallisation this disappeared. In the case of the ester the racemic material was found to crystallise more readily than the optically active material - the second crop of crystals from the mother liquor exhibited a slight rotation and had a melting point of 116.8-117°C compared to 118-118.4°C for the first crop. However the measured rotation was too low to be significant.

Further experiments were carried out with PLE using varying concentrations of DMF as a cosolvent but no reaction was observed at all in these cases.

4.3 INTERESTERIFICATION WITH PLE

The possibility of using an immobilised PLE preparation for an interesterification of the ester (126) with heptanol was considered as an alternative to hydrolysis.

Scheme 52. The interesterification reaction attempted.

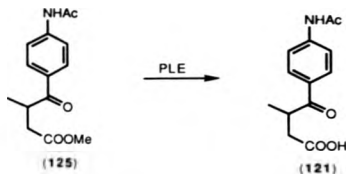


PLE was immobilised on sepharose 4B and the preparation was used for an interesterification reaction using heptanol as both solvent and reagent. The reaction was followed by TLC as the new heptyl ester should be well separated from the initial methyl ester. After 15 days of stirring at room temperature only the starting ester was evident on TLC. A standard assay mixture of ethyl butyrate in phosphate buffer was equilibrated on the autotitrator and some of the enzyme from the reaction mixture was added. No uptake of alkali was observed suggesting that either PLE had been denatured by the heptanol or that it had been washed out of the sepharose.

4.4 HYDROLYSIS OF THE CORRESPONDING AMIDE ESTER WITH PLE

The following reaction was also investigated with PLE.

Scheme 53. Hydrolysis of the protected ester (125).



This reaction was performed in a system comprising phosphate buffer with a 10% ethanol cosolvent. Since the N-acetyl compound is soluble in ethanol whereas the amine compound is not, ethanol was used in preference to methanol to avoid upsetting the equilibrium of the hydrolytic reaction. Again some uptake of alkali was observed before the enzyme was added but this stabilised before the reaction was started (by addition of enzyme). The reaction was stopped at around 50% conversion and ester and acid were extracted as described previously. The rotation of the acid was measured and was found to be zero.

4.5 HYDROLYSIS OF THE METHYL ESTER WITH LIPASES

A screening experiment was performed on the hydrolysis of the methyl ester (126) by various microbial lipases. A stock solution was prepared of 90 mg of the substrate in 9 ml of a solvent system comprising phosphate buffer containing a 10% ethanol cosolvent. This was divided into portions and the enzymes were added. The reactions were stirred at room temperature and monitored by TLC. The enzymes used are detailed below.

Table 9. Lipases used for a screening experiment for hydrolysis of ester (126).

Lipase Source	Supplier	Amount used (mg)
<i>Pseudomonas fluorescens</i>	Amano	19
<i>Mucor javanicus</i>	Biocatalysts	17
<i>Rhizopus javanicus</i>	Biocatalysts	15
<i>Aspergillus</i> sp.	Amano	15
<i>Candida cylindracea</i>	Amano	14
<i>Geotrichum candidum</i>	Biocatalysts	7

A sample of the stock solution was kept as a control - i.e. without enzyme.

The reactions were monitored daily for two weeks and after 2-3 days most had impurities that did not correspond to the acid spot on TLC. The reactions with the first four enzymes in the list showed some acid present after 3-4 days but the reactions were so slow that they were not scaled up.

The hydrolysis was also attempted in a chloroform/water biphasic system. The reactions were each performed on 50mg of the methyl ester (126) with the following enzymes: PPL and microbial lipases from *C. cylindracea*, *A. niger*, *R. javanicus* and *Pseudomonas* sp. Reactions were monitored by TLC of the organic phase but after 7 days no change was observed. It was concluded that lipases are either not active in chloroform or that they simply cannot act upon this substrate.

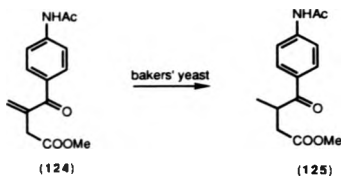
4.6 INTERESTERIFICATION OF THE METHYL ESTER WITH LIPASES

The interesterification in heptanol was attempted using the five lipases as above. The reactions were performed on 20mg of the substrate in heptanol (2ml) as both reagent and solvent. Reactions were followed by TLC for 2 weeks, after which time no change in composition of the solutions was observed. Lipases have previously been shown to be active with heptanol as substrate²⁹⁶ and so it was concluded that our methyl ester was a non substrate for the lipases tested.

4.7 REDUCTION WITH BAKERS' YEAST

As discussed in the introduction in this chapter a second approach to optically active 3-methyl-4-oxo-4-(4-aminobenzyl) butanoic acid involved reduction of the corresponding unsaturated compound with microorganisms.

Scheme 54. Reduction reaction.



It was initially uncertain whether yeasts would perform the desired transformation without concomitant reduction of the carbonyl group and either reduction or hydrolysis of the ester and amide groups. The reduction reaction was initially performed on 100mg of ester (124) using bakers' yeast.

Such fermentations would normally be followed by TLC but in this instance authentic samples of the substrate and product could not be separated in various solvent systems. The incubation was simply worked up after 24h. when carbon dioxide was no longer being evolved. The product was extracted and purified by flash chromatography.

It was confirmed that only the desired transformation had taken place by ^1H NMR of the purified product. Chiral analysis was carried out by measurement of the rotation. The specific rotation of the optically pure ester (126) is reported to be $[\alpha]_{\text{D}} +27.4^\circ\text{C}$.²⁹⁷ Since this closely related compound has a fairly high rotation the product of yeast reduction might be expected to display a similar

figure. However in this instance the products isolated from bakers' yeast reduction displayed specific rotations of only 1-2°. This suggested that although the yeast was evidently performing the desired transformation it was doing so non stereospecifically.

4.8 DEVELOPMENT OF A RELIABLE METHOD OF CHIRAL ANALYSIS

A method for analysis of the optical purity of the reduction products was required since the optical rotation data was not available for this precise compound. Europium chiral shift reagents were found to produce no splitting of the signals on the ¹H NMR of the authentic racemic product. A chiral solvating agent, 1,1,1-trifluoro-1-(9-anthryl)ethanol was found to give a good splitting of the doublet ($\delta=1.2\text{ppm}$) due to the methyl group. This reagent was used for the chiral analysis of products from reduction experiments.

4.9 REDUCTION WITH *CANDIDA GUILLIERMONDII*

The same reduction was attempted with resting cells of the yeast *Candida guilliermondii* NCYC 1399. The yeast was cultivated in flasks on a rotary shaker at 30°C in a medium of Difco YM broth. Previous work had shown that the cells required ~72h growth to reach the early resting stage when they are most efficient for biotransformations.²⁷⁴ Therefore in this instance the substrate was added after 72h. growth. The reaction was monitored by carrying out several fermentations simultaneously and stopping a

reaction every two hours until the conversion was seen to be complete. The reaction of 50mg substrate required 9h. to reach completion with 100ml of the culture. The samples of the product extracted from the completed fermentations all exhibited some rotation. However ^1H NMR analysis of the samples using the chiral solvating agent showed that all of the samples were close to racemic.

Furthermore, a control experiment in which a culture flask not inoculated with the substrate was extracted, showed that some material was coming from the yeast itself. This was found to display a rotation similar to those obtained for the previous products. Hence if this was not properly removed in the chromatographic work up it could easily explain the observed rotations. Thus it appears that *C. guilliermondii* will also perform the desired transformation but again the reaction is non-selective.

4.10 REDUCTION WITH OTHER MICROORGANISMS

Several reductions were performed with strains of *Clostridia*, *Lactobacillus brevis* and *Klebsiella aerogenes*. Because of the problems associated with the toxic properties of the *Clostridia* and the need for an anaerobic fermentation these experiments were kindly performed by Prof. G. Morris at Aberystwyth. The samples isolated from the fermentation broths were returned after removal of the cell debris. The reactions were performed on 100mg substrate and recovery was poor. Each sample received was dissolved in dichloromethane and washed thoroughly with water

to remove remaining biological material. The products were then reisolated by evaporation of the solvent and were analysed by ^1H NMR. Rotations were measured and the chiral analysis was also performed by ^1H NMR using the chiral solvating agent. Results from these experiments are presented below:

Table 10. Results of the reductions performed with bacteria and bakers' yeast

Organism	Degree of reduction	Yield	Rotation	NMR Analysis.
<i>Lactobacillus brevis</i>	No reduction	-	-	-
<i>Clostridium pastorianum</i>	Complete reduction	28mg	0°	rac.
<i>Clostridium tyrobutyricum</i> *	Complete reduction	16mg	-0.5°	rac.
<i>Clostridium kluyveri</i> **	Some product, much additional material	12mg	-	-
<i>Klebsiella aerogenes</i>	Mostly product. Some substrate and extra material	33mg	+0.46°	rac.
<i>Saccharomyces cerevisiae</i>	No reduction	-	-	-
<i>Clostridium trybutyricum</i> **	Complete reduction but extra material present	11mg	-	-

* Grown on glucose

** Grown on crotonate

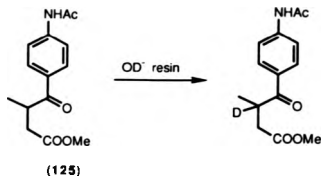
It can thus be seen that all of the *Clostridia* actually reduce the substrate to the desired product but with no selectivity. It is

interesting to note that in this instance the *Saccharomyces cerevisiae* did not effect the reduction at all whereas the strain obtained from a local supermarket did.

4.11 INVESTIGATION OF LABILITY OF THE PRODUCT UNDER FERMENTATION CONDITIONS

At this point it was considered worthwhile investigating whether the product could be labile under the conditions of fermentation i.e. whether it could be spontaneously racemising after formation. A sample of the product was deuterated by stirring in d_1 methanol with a deuterated anion exchange resin prepared by exchange of D_2O with the OH^- form of the resin. The effect of the resin on the product was studied by 1H NMR.

Scheme 55. Deuteration of the racemic compound (125).



The methyl doublet (produced by coupling to the exchanged proton) was gradually seen to collapse to a singlet over 12 hours. At the same time the two double doublet resonances due to the prochiral methylene group gradually became two doublets. A

small amount of non deuterated material remained as shown by 400MHz ^1H NMR. At equilibrium (after 48h. when no further change was observed) the mixture contained a ratio of deuterated: non deuterated material of approximately 6:1. The deuterated material was isolated by filtering off the resin and washing this with a little d_1 methanol. The solvent was then evaporated. Fermenting bakers' yeast was prepared as in previous experiments and this was inoculated with the deuterated product. The mixture was stirred at 30°C for 24h. after which time the material was reisolated by the same procedure as used earlier. After column chromatography and recrystallisation ^1H NMR of the isolated material showed that it was still deuterated. This proves that the proton at the chiral centre, adjacent to the carbonyl group is not labile under the conditions of the fermentation, and therefore that the reduction is non-stereospecific.

4.12 SUMMARY AND CONCLUSIONS

Although the various hydrolytic procedures attempted on the methyl ester were full of problems with unaccountable uptake of alkali from the autoburette and with product purification it can be seen that PLE will actually hydrolyse the methyl ester. The only method of chiral analysis used in the hydrolytic reactions was measurement of rotation but the expected product should have a specific rotation of $+27^\circ$ (for the (R)-(+)-enantiomer) and so this should be large enough to give some measure of enantioselectivity by measurement of the rotation. Hence it can be concluded that

PLE was non-selective in its hydrolysis of this compound. The hydrolysis with the lipases tried did not appear promising as none of the enzymes was very active on the substrate.

Interesterification was similarly unsuccessful.

The reductions would have proved to be an extremely profitable reaction since starting from an achiral substrate theoretically a 100% yield of the optically pure material is available. However, despite the fact that a variety of microorganisms was used racemic products were always obtained. Furthermore, it was proved that the compound does not racemise under the fermentation conditions, showing that the reaction is non-stereospecific.

CHAPTER 5

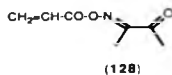
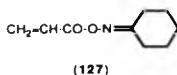
RESOLUTION OF KETONES VIA HYDROLYSIS OF A CORRESPONDING OXIME ESTER.

5.1. INTRODUCTION.

There are no examples in the literature of the resolution of ketones *per se* using biotransformations. A novel approach to the production of optically active ketones lies in the kinetic resolution of their corresponding oxime esters via enzyme-catalysed hydrolysis. This may then be followed by chemical hydrolysis of the optically active oxime to give the ketone.

Only two papers are to be found in the literature of biotransformations involving oximes. In the first of these, oxime esters were used as irreversible acyl transfer agents in transesterification reactions catalysed by PPL.²⁹⁸

Scheme 55. Oxime esters as acyl transfer agents.



The oxime esters (127, 128) were used and a variety of alcohols was resolved using this method.

Oxime esters were more reactive than alkyl- or enol esters and oxime acetates were more reactive than oxime acrylates.

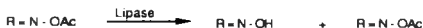
In the second example oxime esters were again used as irreversible transesterification reagents, this time in the production of chiral acrylate polymers.²⁹⁹

Scheme 56. Oximes in transesterification.



The aim of the investigations described below was to attempt the novel kinetic resolution of oxime esters.

Scheme 57. Resolution of oxime esters.



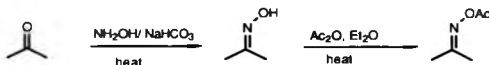
Optically active ketones may then be readily obtained via cleavage of the oxime.

5.2 PREPARATION AND HYDROLYSIS OF ACETONE OXIME ACETATE.

At the time that this work began there were no reported uses of oxime esters in the literature of biotransformations. Therefore acetone oxime acetate was prepared and enzymatically hydrolysed to show that enzymes could actually act upon these substrates.

The oxime ester was prepared by reported methods, starting from acetone itself.^{300,301}

Scheme 58. Preparation of acetone oxime acetate.



The oxime was isolated and identified by spectroscopic techniques prior to conversion to the oxime acetate.

The oxime acetate was subjected to hydrolysis with pig liver esterase in phosphate buffer at pH 7.0 controlled by a pH-stat.

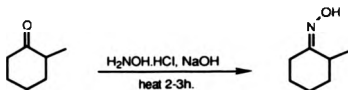
The uptake of alkali from the autoburette showed that complete hydrolysis occurred. Upon extraction the oxime was obtained as expected. No further hydrolyses were attempted with other enzymes but it had been shown that pig liver esterase accepted an oxime ester as substrate.

5.3 PREPARATION AND HYDROLYSIS OF

2-METHYLCYCLOHEXANONE OXIME ACETATE.

The first chiral ketone chosen for study was 2-methylcyclohexanone. The oxime was synthesised according to a published procedure.³⁰²

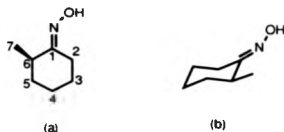
Scheme 59. Preparation of 2-methylcyclohexanone oxime.



This was isolated and purified by recrystallisation from hexane at dry ice temperature. The melting point of the product was as previously reported for the oxime³⁰³⁻³⁰⁵ and it was analysed by

spectroscopic techniques. There have been several reports that the oxime of 2-methylcyclohexanone is formed exclusively as the isomer shown in Fig. 61a.^{306,307}

Figure 61. Conformation of 2-methylcyclohexanone oxime.



In addition, detailed ^1H NMR studies have shown the conformation to be E- equatorial (Fig. 61b).

In a further study the ^{13}C NMR chemical shifts of a large number of ketones and their oximes have been assigned.³⁰⁸ In this study the oxime of 2-methylcyclohexanone was prepared by treatment of the ketone with hydroxylamine (as above) and was found to contain 84% of the *anti* isomer shown above. ^{13}C NMR chemical shifts were assigned for this and the minor *syn* isomer. In this study it was suggested that the steric influence of the hydroxyl and 2-methyl groups in the minor isomer brought about a more or less complete conformational change, and hence the methyl group was axial. Despite repeated recrystallisation the ^{13}C NMR spectrum of the sample of the oxime obtained consistently showed that it contained two components. Based upon the data in the study described above³⁰⁸ both major and minor compounds were readily identified as the *anti* and *syn* isomers of the oxime, as anticipated. The ^{13}C NMR data are shown in Table 11.

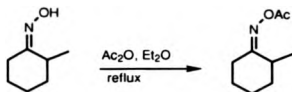
Table 11. Comparison of predicted ^{13}C NMR chemical shifts (δ_{pred})³⁰⁸ for major and minor isomers of 2-methylcyclohexanone oxime with those observed (δ_{obs}) in the current work.

C	major (anti) isomer		minor (syn) isomer	
	δ pred.	δ obs.	δ pred.	δ obs.
C-1	163.0	163.1	163.6	—
C-2	37.2	37.1	26.8	26.6
C-3	35.7	35.5	26.7	26.5
C-4	23.9	23.7	20.5	20.3
C-5	24.8	24.6	28.4	28.2
C-6	26.1	25.9	31.7	31.5
C-7	16.9	16.7	16.2	16.0

Based on the peak heights the mixture was seen to contain 28% of the minor isomer.

The oxime acetate was formed from this mixture by the same procedure as described for formation of acetone oxime acetate.

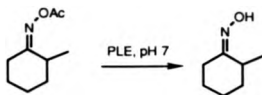
Scheme 60. Preparation of the oxime acetate.



It was purified by flash chromatography and identified by its spectroscopic data. Study of the ^{13}C NMR spectrum showed that the amounts of the configurational isomers changed on acetylation, with the proportion of the minor isomer increasing relative to the major product.

This oxime acetate was hydrolysed using PLE in a phosphate buffer maintained at pH 7 by addition of sodium hydroxide solution from an autoburette.

Scheme 61. Hydrolysis of the oxime acetate.



The consumption of alkali went to the theoretical 100% without a rate change being observed.

A screening experiment was then performed with lipases.

Reactions were performed by addition of various lipases (15-30 mg) to aliquots of a stock solution comprising 200 mg of the oxime acetate in 20 ml phosphate buffer containing 1 ml methanol (to give a homogenous solution). After addition of the enzymes the solutions were stirred at room temperature and reactions were followed by TLC. The solvent system used was ether:petrol 1:1 which was shown to give a clean separation of authentic samples of oxime and oxime acetate.

The enzymes used are detailed below (Table 12)

Table 12. Enzymes used in screening for hydrolysis of 2-methylcyclohexanone oxime acetate.

expt	lipase source	supplier	amt*
A	<i>Mucor</i>	Amano	28
B	<i>Candida cylindracea</i>	Amano	30
C	<i>Pseudomonas</i>	Amano	30
D	<i>Aspergillus</i>	Amano	23
E	<i>Penicillium cyclopium</i>	Biocatalysts	22
F	<i>Penicillium roqueforti</i>	Biocatalysts	16
G	<i>Geotrichum candidum</i>	Biocatalysts	21
H	<i>Candida lipolytica</i>	Biocatalysts	15
I	<i>Rhizopus delemar</i>	Biocatalysts	15

*amount (mg) per 20 mg substrate.

A control experiment (with no enzyme) showed no hydrolysis. After stirring for 36 h. at room temperature several of the reaction mixtures were found to contain both oxime and oxime acetate, suggesting that the reactions had slowed down or stopped altogether after hydrolysis of one enantiomer of the substrate. A reliable method of chiral analysis was sought at this stage before scaling up the reactions. Investigation of the ^1H NMR of the racemic oxime acetate upon addition of two equivalents of (S)-(+)-2,2,2-trifluoro-1-(9-anthryl)ethanol showed even splitting of the doublet due to the 2-methyl group, and this was selected as a criterion of optical purity. Four lipases were selected and the hydrolysis was scaled up to the use of 200 mg oxime acetate with each of these. Reactions were again performed in phosphate buffer with a small amount of methanol as a cosolvent. The conversion was monitored at 30 min intervals by ether extraction of small samples and GLC analysis.

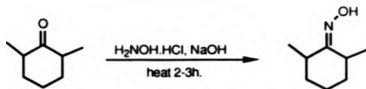
The lipase from *Candida cylindracea* was found to give complete conversion within the first 30 min and so was excluded from further work. The reactions with lipases from *P. roqueforti*, *P. cyclopium* and *R. niveus* were worked up when the GLC peaks due to oxime and oxime acetate were approximately of equal area. The products and remaining starting material were extracted and the compounds separated by flash chromatography. The recovered acetates were analysed by NMR and then subjected to chiral analysis by ^1H NMR in the presence of 2 equivalents of (S)-(+)-2,2,2-trifluoro-1-(9-anthryl)ethanol. In each case the methyl signal was observed to split into equal doublets, showing that the samples were racemic.

It was therefore concluded that these lipases were completely non-stereoselective for reaction on this substrate.

5.4 PREPARATION OF 2,6-DIMETHYLCYCLOHEXANONE OXIME ACETATE.

The oxime of 2,6-dimethylcyclohexanone was prepared as described for 2-methylcyclohexanone, and was isolated as colourless, needle-like crystals.

Scheme 62. Preparation of 2,6-dimethylcyclohexanone oxime.



The oxime was prepared from commercially available 2,6-dimethylcyclohexanone which is a mixture of *cis* and *trans*

isomers. ^1H NMR of the oxime obtained after recrystallisation shows that either a small trace of ketone or of a second, minor isomer is present as a second set of methyl resonances is just evident on the baseline next to the major set. The ^{13}C NMR spectrum however was clean. Geneste *et al*³⁰⁹ have reported on the stereochemical analysis of oximes using ^{13}C NMR spectroscopy. In this study the ^{13}C NMR data from a number of relatively rigid oximes was compared to similar data for the parent ketones. Using these models the ^{13}C NMR chemical shifts were predicted for the *cis* and *trans* isomers of 2,6-dimethylcyclohexanone based upon spatial interactions calculated from the methyl group interactions and the shielding effect and methyl parameters for the cyclohexane derivatives. Results with authentic compounds are reported to show that the chemical shifts were predicted reasonably well. Geneste's predicted and observed ^{13}C NMR data are summarised in Table 13.

Table 13. Predicted values for the ^{13}C NMR shifts of the *cis* and *trans* isomers.

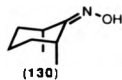
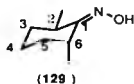


Table A *trans* isomer (129)

C	δ_{pred}	Δ
2	26.6	-0.1
3	32.1	+0.1
4	19.9	-0.7
5	36.1	0
6	33.2	0

Table B *cis* isomer (130)

C	δ_{pred}	Δ
2	26.6	-0.7
3	32.1	+1.0
4	15.4	-1.0
5	33.1	+1.3
6	33.2	-0.8

$$\Delta = \delta_{\text{predicted}} - \delta_{\text{observed}} \text{ (reported)}^{309}$$

The table below shows the comparison of Geneste's predicted values for the *cis* isomer with the data obtained for the sample in question.

Table 14. Predicted and experimental ^{13}C NMR shifts for the ring carbon atoms of 2,6-dimethylcyclohexanone oxime (major isomer).

C	$\delta_{\text{pred}}(\text{ppm})$	$\delta_{\text{obs.}}(\text{ppm})$	$\delta_{\text{pred}} - \delta_{\text{obs.}}$
C-1	167 ± 1	166.4	0.6
C-2	26.6	27.1	-0.5
C-3	32.1	30.8	1.3
C-4	15.4	16.1	-0.7
C-5	33.1	31.4	1.7
C-6	33.2	33.8	-0.6

$$\delta_{\text{pred}} = \text{calculated values}^{309}$$

δ_{obs} = observed values for the sample prepared here.

It may be seen that the shift values observed previously differed by approximately the same margins as in this case. This configuration may be confirmed by study of the ^1H NMR spectrum. PC MODEL (Serena software) may be used to calculate energy minima and thus the conformations in which both *cis* and *trans* isomers of this compound are likely to exist. Minimising the structures for the *cis* diaxial and *cis* diequatorial conformations shows that both minimise to a slightly distorted chair conformation as shown in Fig. 62a,b. The energies of these structures are 20.98 Kcal mol $^{-1}$ and 23.48 Kcal mol $^{-1}$ for the diaxial and diequatorial structures respectively. The *trans* isomer minimises to a similar value and is again a chair conformer.

Figure 62. Minimised conformations. (a) *cis* diaxial, (b) *cis* diequatorial, (c) *trans* conformations.

Figure 62a.

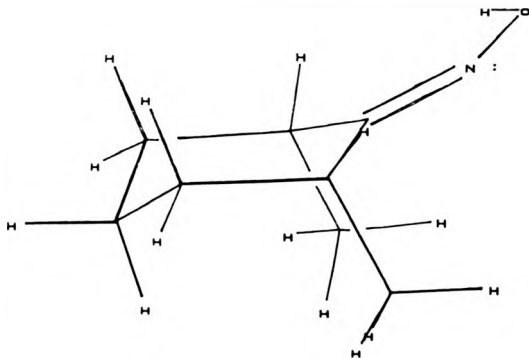


Figure 62b

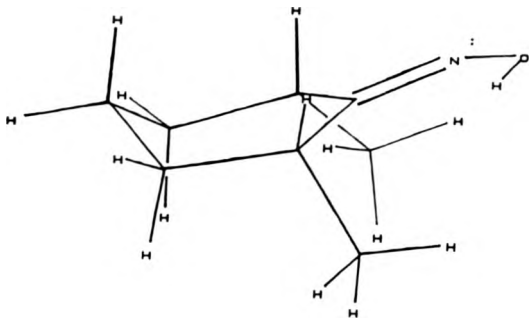
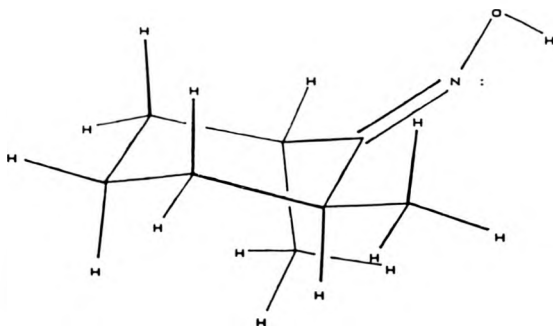


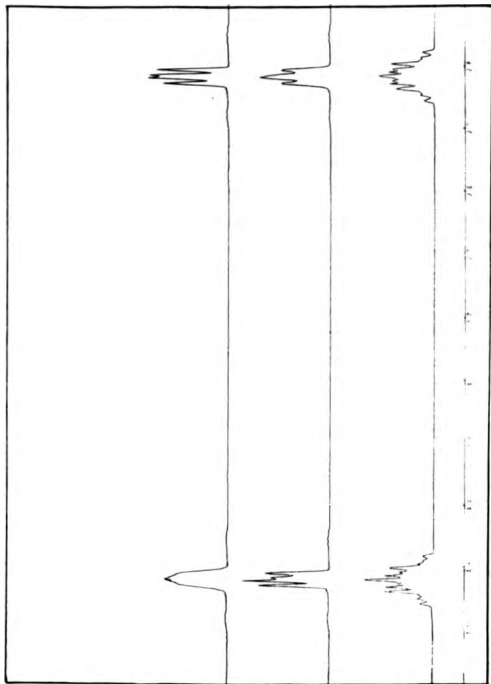
Figure 62c.



Coupling constants were calculated for the C-2 and C-6 protons to the adjacent C-3 and C-5 vicinal pair for each of the three structures. It is immediately apparent that there is a large difference in the coupling constants between the *cis* and *trans* configurations, as would be expected from the appearance of the structures. The *trans* isomer is expected to have a large (12.36 Hz) and a small (3.02 Hz) vicinal coupling for the proton adjacent to the equatorial methyl group. The proton adjacent to the axial methyl group should display coupling constants of 4.67 Hz and 1.86 Hz.

The decoupled ^1H NMR spectrum was obtained by irradiation at each of the methyl signals in turn. Each of the signals due to the C-2 and C-6 was seen to break down into a set of 4 peaks with no apparent large coupling (see Fig. 63). Thus the *trans* isomer was ruled out and it remained to be seen whether the conformation formed was the *cis* diaxial or *cis* diequatorial. The two *cis* conformations appear very similar. However calculation of the dihedral angles around the C-2 and C-6 protons show that the *cis* diaxial conformation is symmetrical with both methyl groups oriented pseudoaxially. The dihedral angles of the minimised *cis* diequatorial structure however, show that this half chair conformation is asymmetrical with one methyl group pseudoaxial and the other pseudoequatorial.

Figure 63. ^1H NMR spectrum of the oxime decoupled at C-2 and C-6 in turn.



The computer programme can also be used to simulate the ^1H NMR spectrum of parts of the molecule in question, given coupling constants and chemical shift values of the protons concerned. This experiment was performed on the two minimised structures using the C-2 - C-3 coupling constants derived above and assigning various values to the unknown geminal coupling between the C-3 protons. Use of various arbitrary values showed that this coupling had little influence on the generated spectrum. The table below gives frequencies and relative intensities of the peaks generated for the C-2 proton of the two structures, using a geminal coupling constant of 18 Hz.

Table 15. Simulated ^1H NMR data for the two *cis* conformations.

Diaxial		diequatorial	
freq(Hz)	rel. int	freq(Hz)	rel. int
1342	0.01	1341.9	0.015
1357	1.01	1351.4	1.025
1360	1.97	1360.0	0.986
1365	0.98	1360.11	0.985
		1368.7	0.975
		1378.2	0.014

rel. int = relative intensity.

The decoupled spectra for the actual molecule are shown in Fig 63. It may be seen that the decoupling produced a set of four peaks of roughly equal height.

Study of these spectra shows that in the first instance the simulation of the diequatorial structure is most closely matched because it produces four peaks of approximately equal height. The problem with this is that the differences in frequency

between the peaks do not match those on the observed spectrum.

The frequencies and relative intensities of the peaks observed are shown below.

Table 16. Frequencies and relative intensities of the observed peaks.

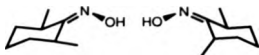
decoupled at 1.1878 ppm		1.1756 ppm	
freq (Hz)	rel int.	freq (Hz)	rel int.
1373.1	4.21	1052.3	3.73
1370.2	5.17	1048.7	4.68
1367.9	3.47	1047.0	4.55
1365.0	3.83	1043.5	4.13

The difference in frequencies between the two central peaks are clearly larger in the simulated spectrum. However, the difference in frequencies of the central peaks differs between the two decouplings (2.2 Hz for decoupling at 1.1878 ppm and 1.7 Hz for decoupling at 1.1756 ppm). This is a difference of approximately 25% suggesting strongly that the molecule is asymmetric.

Thus it may be said with a reasonable degree of certainty that the conformation of the 2,6-dimethylcyclohexanone formed is *cis* diequatorial.

It is important to note that with the molecule in this conformation the only source of chirality is the oxime group itself.

Figure 64. The enantiomers of 2,6-dimethylcyclohexanone oxime.



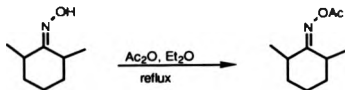
Therefore the oxime may be resolved via enzymatic hydrolysis of an ester but cleavage of the oxime will not generate a chiral ketone. Although this means that this molecule is no longer useful for the original aim of this work, this provides an elegant example of the use of molecular modelling to determine the shape of the molecule in question.

A study has been reported on the equilibrium concentrations of the different configurational isomers of 2,6-dimethylcyclohexanone oxime.³¹⁰ The *cis* and *trans* isomers were each equilibrated at 80°C in ethanol for three weeks using a small amount of hydroxylamine acetate as catalyst. The mixtures resulting from heating of the *cis* and *trans* isomers respectively contained 82% and 80% of the *trans* isomer, although details were not provided as to how the relative concentrations were calculated, the authors merely stating that this was done by NMR.

This is apparently contradictory to the fact that the sample in question seems to be the *cis* diequatorial isomer.

The oxime acetate was formed from the oxime by heating with acetic anhydride and ether, as for 2-methylcyclohexanone.

Scheme 63. Preparation of the oxime acetate.



The compound was purified by flash chromatography and identified by spectroscopic techniques. ^1H and ^{13}C NMR spectra were as anticipated, again suggesting that only one isomer had been formed.

5.5 ENZYMATIC HYDROLYSIS OF THE OXIME ACETATE.

The oxime acetate obtained in the previous section was then subjected to enzymatic hydrolysis. A screening experiment was performed with lipases in an aqueous system. The following enzymes were used (amounts given = mg per 10 mg substrate).

Table 17. Enzymes used in the screening for hydrolysis of the oxime acetate.

lipase source	supplier	amt	result after 36h.	
			P	S
<i>Rhizopus</i> sp.	Amano	13	+	-
<i>Candida cylindracea</i>	Amano	29	+	-
<i>Pseudomonas</i> sp.	Amano	26	+	-
<i>Aspergillus</i> sp.	Amano	12	+	-
<i>Mucor</i> sp.	Amano	14	+	-
<i>Geotrichum candidum</i>	Biocatalysts	9	+	-
<i>Penicillium cyclopium</i>	Biocatalysts	16	+	+
<i>Rhizopus delemar</i>	Biocatalysts	23	-	+
<i>Mucor javanicus</i>	Biocatalysts	18	+	+
<i>Penicillium roqueforti</i>	Biocatalysts	14	+	+
<i>Rhizopus arrhizus</i>	Biocatalysts	9	-	+
<i>Candida lipolytica</i>	Biocatalysts	11	-	+
<i>Pseudomonas fluorescens</i>	Biocatalysts	10	+	-
<i>Rhizopus niveus</i>	Biocatalysts	10	+	+
<i>Rhizopus javanicus</i>	Biocatalysts	16	+	+
No enzyme	-	-	-	+

P = product, S = substrate.

The TLC system used to detect the oxime and oxime acetate in this screening experiment was not as successful as expected in the separation of the spots. Some experiments were repeated on a 50 mg scale on the autotitrator using lipases chosen from the screening experiment.

With lipases from *R. javanicus*, *P. roqueforti* and *R. arrhizus* no alkali uptake was observed at all. Reactions with lipases from *R. niveus*, *C. lipolytica* and *P. cyclopium* appeared to slow down toward 50% conversion. These reactions were worked up by extraction and samples of oxime and oxime acetate were obtained after preparative TLC. Rotations were measured and were found to be insignificant in each case. This was not however unexpected since oximes do not generally exhibit high rotations.

Further experiments were performed using a higher enzyme : substrate ratio (1.5:1 instead of 1:1). The reactions were allowed to run to completion to investigate the shapes of the curves due to alkali uptake from the autoburette. The lipase from *Rhizopus niveus* showed anomalous results with a short time of steady uptake of alkali followed by a constant rapid uptake. The reaction was stopped at an apparent 200% conversion as judged by the alkali consumption and TLC of the reaction mixture showed that both oxime and oxime acetate were present. This experiment was performed twice on different titrators and the same result was obtained. This result is believed to be due to either dissolved carbon dioxide in the reaction mixture or to some other hydrolysable material present in either the substrate or the enzyme preparation.

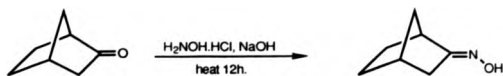
Reactions with lipases from *P. cyclopium* and *C. lipolytica* showed little rate change at the 50% conversion. To confirm this result the hydrolyses were performed again on a larger scale and were worked up at approximately 50% conversion as judged by alkali uptake. It is believed that in this instance this will be reasonably accurate because the reactions are fast, allowing little chance for the result to be affected by carbon dioxide dissolving from the atmosphere.

^1H NMR experiments on the racemic oxime using two equivalents of the chiral solvating agent (S)-(+)-2,2,2-trifluoro-1-(9-anthryl)ethanol gave equal splitting of the two methyl doublets. This was therefore considered to be a criterion for measurement of the optical purity of the oxime. The reaction mixtures were extracted, the product was separated and the oximes were analysed by ^1H NMR using the chiral solvating agent. The splitting showed that the products were racemic.

5.6 PREPARATION AND ENZYMATIC HYDROLYSIS OF NORCAMPHOR OXIME ACETATE

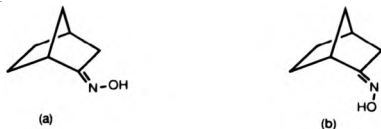
Norcamphor oxime was prepared by the method described previously for 2-methylcyclohexanone and 2,6-dimethylcyclohexanones except that the heating period was extended to approximately 12h., (or overnight) to achieve the desired conversion.

Scheme 64. Preparation of norcamphor oxime



The oxime has been characterised in earlier work as an oil boiling at 114-116°C at 12 mmHg. The product obtained in this work was purified by distillation and gave a clear oil boiling at 105°C at 1 mmHg. The oil solidified upon standing to give a colourless solid of melting point 50.2-51.6°C. Further characterisation was by NMR spectroscopy. The ^1H NMR spectrum of this type of compound is extremely difficult to interpret in detail, owing to the complicated overlapping resonances. However, the resonances obtained agree with what would be expected. The ^{13}C NMR spectrum has been fully interpreted for both of the possible configurational isomers.³⁰⁸

Figure 65. The two configurational isomers of norcamphor oxime.



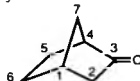
The ^{13}C NMR spectrum of the norcamphor oxime obtained clearly shows the presence of two isomers. The major component of the mixture may be identified readily as the oxime with the configuration shown in fig 65a, due to the close correlation of the data with that observed by Hawkes *et al.*³⁰⁸ This data is shown in the table below. δ_{pred} are the chemical shifts observed by Hawkes and δ_{obs} are the experimentally determined data.

Table 18. Correlation of the data reported by Hawkes with experimentally determined data for the major oxime isomer.

C	$\delta_{\text{pred}}(\text{ppm})$	$\delta_{\text{obs}}(\text{ppm})$	$\delta_{\text{pred}} - \delta_{\text{obs}}$
C-1	42.0	42.15	-0.15
C-2	167.4	167.7	-0.32
C-3	34.9	34.7	0.24
C-4	35.5	35.29	0.21
C-5	27.1	26.90	0.20
C-6	27.8	27.61	0.19
C-7	39.1	38.89	0.21

The minor compound is present as less than 20% of the total material. These resonances may be tentatively assigned to the parent ketone as shown in Table 19.

Table 19. Assignment of the ^{13}C NMR signals of norcamphor



C	$\delta_{\text{pred}}(\text{ppm})$	$\delta_{\text{obs}}(\text{ppm})$	$\delta_{\text{pred}} - \delta_{\text{obs}}$
C-1	49.3	47.71	1.59
C-2	216.8	199.31	17.49
*C-3	44.7	-	-
C-4	34.8	37.07	-2.27
C-5	26.7	27.20	-0.50
C-6	23.7	25.78	-2.08
C-7	37.1	38.16	-1.06

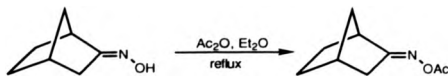
* This resonance might accidentally coincide with the C-1 resonance of the major isomer.

It was concluded from this information that the oxime was comprised of a mixture of 80% of the desired compound in the configuration shown and 20% of the parent ketone.

The mass spectrum showed the parent ion peak at m/z 125 as expected and various fragments and a small peak at m/z 110, possibly attributable to the ketone.

The corresponding oxime acetate was prepared from this oxime by the standard method of heating under reflux with acetic anhydride in ether.

Scheme 65. Preparation of norcamphor oxime acetate.



The crude product was isolated and purified by flash chromatography. The spectral data for this compound were as expected, although an impurity (five resonances ca. 20% of the peak height of the major peaks) was observed in the ¹³C NMR spectrum which could not be identified as either oxime or ketone.

A screening experiment with nine lipases for the hydrolysis of this compound was performed and showed that the reactions were catalysed readily by most of the enzymes tested. With lipases from *Candida cylindracea* and *Aspergillus niger* the hydrolyses were complete within 4h. Lipases from *Pseudomonas* sp., *Candida lipolytica*, *Mucor javanicus* and *Penicillium cyclopium* were selected for further study because some starting material still remained in their screening experiments after 18h. It should be noted that a control experiment was performed in the screening work and that a trace of product was evident in this experiment after 18h.

Prior to scaling up the hydrolysis a reliable method of chiral analysis of the product was sought. It has already been reported that measurement of enantiomeric excess of norcamphor directly using GLC or HPLC analysis of a variety of derivatives by ^1H NMR or ^{13}C NMR with shift reagents was unsuccessful.³¹¹ It therefore seemed unlikely that the situation with the oxime would be any different. Attempts to obtain a splitting of the acetate peak of the oxime ester using (S)-(+)-2,2,2-trifluoro-1-(9-anthryl)ethanol were unsuccessful. The most suitable method of chiral analysis therefore was deemed to be hydrolysis of the oxime to the ketone, and measurement of the rotation.

The oxidative cleavage of the oxime was performed in dichloromethane with aqueous bromine, buffered with sodium hydrogen carbonate under mild conditions at room temperature.³¹²

Scheme 66. Cleavage of the oxime.



Sodium hypobromite, generated *in situ* is believed to be the reagent responsible for the reaction which has previously been demonstrated to give a high yield of norcamphor from the oxime.³¹²

The lipase-catalysed hydrolyses were then scaled up using the enzymes selected from the screening work and were performed on an autotitrator. The hydrolyses with lipases from *P.*

cyclopium and *M. javanicus* gave smooth curves with no rate change at any stage. The reaction with lipase from *Pseudomonas* sp. on the other hand slowed down dramatically after consumption of 50% of the theoretical amount of sodium hydroxide solution from the autoburette. The reaction was also attempted on the autotitrator with pig liver esterase and a very rapid uptake of alkali was observed, stopping after complete hydrolysis. Reaction with subtilisin Carlsberg was observed to give a smooth consumption of alkali, also stopping at 100% hydrolysis. Norcamphor oxime acetate proved, however to be a non-substrate for α -chymotrypsin, as no alkali was consumed at all.

The hydrolysis with lipase from *Pseudomonas* sp. was performed on a preparative scale (200 mg substrate) and was worked up when 47% of the theoretical amount of alkali had been consumed. The product and unreacted starting material were extracted and separated by flash chromatography. The recovered oxime was cleaved using aqueous bromine for chiral analysis. In our hands however, working on a small scale the ketone was formed but it proved impossible to obtain crystalline norcamphor from this reaction. Detection of the ketone on TLC was difficult due to its volatility and for the same reason removal of impurities under high vacuum was impractical.

The rotation of the crude ketone was measured and the specific rotation was found to be $[\alpha] + 8.06$ (c 1.26% in CHCl_3). The rotation of enantiomerically pure norcamphor has been reported to be $[\alpha]_{\text{D}} + 28.3^\circ$ (c 5.3% in CHCl_3)³¹³ or $[\alpha]_{\text{D}} + 15^\circ$ (in CHCl_3)³¹⁴ or $[\alpha]_{\text{D}} + 17^\circ$ (in CHCl_3).³¹⁵ Thus although the ketone

was impure and the true value of the rotation is uncertain it appears that the reaction was stereoselective.

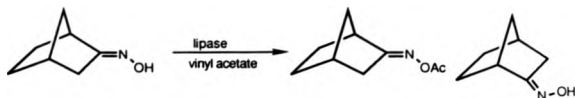
To eliminate these uncertainties in subsequent experiments the ketone was isolated as its 2,4-dinitrophenylhydrazine (DNP) derivative. It has been demonstrated previously³¹¹ that the rotation of the pure enantiomer of the DNP derivative of norcamphor was $[\alpha]_D -51.3^\circ$ (c 2.8 in CHCl_3). The hydrolysis was stopped at approximately 50% conversion as judged by alkali consumption and the oxime remaining was isolated and cleaved to the ketone. The specific rotation of the DNP derivative of norcamphor derived from the hydrolysis product was $[\alpha]_D +14^\circ$ (c 1.3 in CHCl_3). This represents an enantiomeric excess of 27.5%.

This experiment was repeated and was again stopped at ~50% hydrolysis. The oxime was isolated and converted into its DNP derivative as described previously. The rotation of the DNP-norcamphor was $[\alpha]_D +8.6^\circ$ (c 1 in CHCl_3).

In order to improve this selectivity the reaction was attempted using lipase SAM II which as stated earlier is believed to be a more highly purified preparation of lipase P. The reaction was performed on a preparative scale (500 mg substrate) and was stopped after 41% conversion (as judged from the uptake of alkali from the autoburette). The reaction was worked up as described previously and the ketone was again isolated as its DNP derivative. The DNP derivative isolated had a slightly depressed melting point, 125-126°C compared to 129-130°C and had a rotation of $[\alpha]_D +2.6^\circ$. This thus shows that the purified lipase SAM II gives a lower selectivity than lipase P.

The transesterification of norcamphor oxime was also attempted using lipase SAM II and vinyl acetate.

Scheme 66. Transesterification of norcamphor oxime.

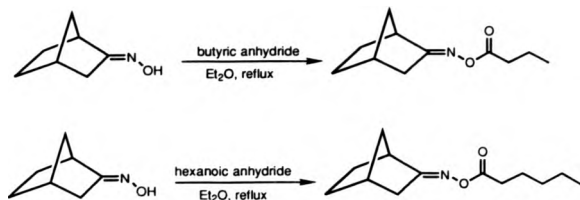


The lipase was simply added to a solution of the substrate and 3 equivalents of vinyl acetate dissolved in *tert.* butyl methyl ether. A duplicate reaction mixture was prepared without enzyme, as a control. Both mixtures were stirred at room temperature and reaction was followed by TLC. The product oxime acetate was observed in roughly equal amounts in both the lipase-catalysed reaction and in the control. This shows that this reaction was unsuitable for resolution purposes.

5.7 PREPARATION AND ENZYMATIC HYDROLYSIS OF NORCAMPHOR OXIME BUTANOATE AND HEXANOATE.

Two further esters of norcamphor oxime were prepared and hydrolysed with lipase P, again in an attempt to improve the enantioselectivity. Norcamphor oxime butanoate and hexanoate were prepared by heating an ethereal solution of the oxime under reflux with the appropriate acid anhydride. Both reactions were followed by GLC and a new peak was observed on each chromatogram. Reactions were worked up after disappearance of the starting material was observed (2.5-3.0h.).

Scheme 67. Preparation of the oxime esters.



Both of these compounds were identified by spectroscopic techniques and their composition was confirmed by elemental analysis.

The enzymatic hydrolyses were performed on an autotitrator using lipase P. Both reactions proceeded rapidly with no rate change at 50% hydrolysis, unlike that observed with the corresponding acetate.

Controls without enzyme for both hydrolyses showed a very slow uptake of the alkali showing that a slow spontaneous hydrolysis was occurring.

It was concluded that lengthening the acid chain increased the rate of reaction but reduced the enzyme's ability to recognise the chirality in the other part of the molecule.

5.8 CONCLUSIONS.

The hydrolyses of the oxime acetates of the ketones chosen were shown to proceed readily when catalysed by various lipases. Experiments with the oxime acetates of 2-methylcyclohexanone and 2,6-dimethylcyclohexanone, however

showed that the hydrolyses with the lipases used were non-stereoselective. In the example of norcamphor oxime acetate, some enantioselectivity was observed in the hydrolysis catalysed by lipase from *Pseudomonas* sp. (lipase P). The DNP derivative of the norcamphor obtained from these experiments was shown to have an enantiomeric excess of 27%. Attempting to improve this by using a more highly purified enzyme preparation resulted in loss of the enantioselectivity. Using the corresponding oxime butanoate and oxime hexanoate esters resulted in complete loss of stereoselectivity. The results obtained suggest that it may be possible to resolve certain ketones using this method. It will however, be necessary to screen many more potential substrates with the lipases. It appears that the acetate is the ester most likely to give high selectivity. Since the lipase finds these esters more difficult to hydrolyse than longer chain esters, the reaction is slower and the enzyme is more likely to recognise the chirality of the molecule.

CHAPTER 6

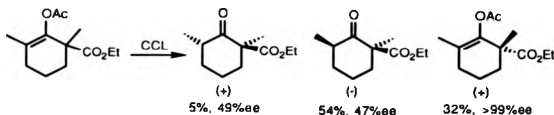
RESOLUTION OF CHIRAL KETONES VIA ENZYMIC HYDROLYSIS OF AN ENOL ACETATE

6.1 INTRODUCTION

A second method of resolution of ketones has been investigated. This involved enzymatic hydrolysis of the enol acetate derived from the chiral ketone. Although various enol acetates and other enol esters have been extensively used as acyl donors in transesterification reactions only two reports have been found in the literature of hydrolysis of an enol acetate.

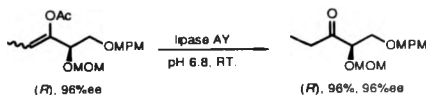
In the first of these an optically active enol acetate was obtained from a mixture of diastereomeric enol acetates,¹⁷⁸ by hydrolysis with lipase from *Candida cylindracea*.

Scheme 68. Resolution of an enol acetate



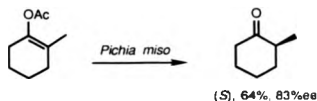
In the second the lipase was simply used to provide a mild method of cleavage of an optically active enol acetate without racemisation.¹⁷⁷

Scheme 69. Hydrolysis of an optically active enol acetate.



Enol acetates have been resolved using whole microorganisms and the substrate in Scheme 69 was the substrate remaining from an enantioselective hydrolysis catalysed by a culture of *Bacillus coagulans* KU 5185. The hydrolysis and stereospecific protonation of the enol acetate of 2-methylcyclohexanone has been performed by a microorganism.³¹⁶

Scheme 70. Hydrolysis of the enol acetate of 2-methylcyclohexanone.



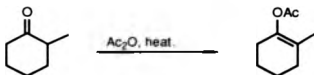
Use of a propionate ester in place of the acetate produced (S)-2-methylcyclohexanone in 98% yield, 94% ee. This resolution was attempted with isolated enzymes during the course of the investigations described below. Much of the work described on this chapter had already been performed at the time of publication of the last report and so it will still be described here. The resolution of 2,6-dimethylcyclohexanone and norcamphor are also considered using this procedure.

6.2 RESOLUTION OF 2-METHYL CYCLOHEXANONE

6.2.1 EXPERIMENTS WITH THE NON-CHIRAL ENOL ACETATE

The non chiral isomer of the enol acetate of 2-methylcyclohexanone was prepared by a well documented procedure.³¹⁷

Scheme 71. Preparation of the non chiral isomer of the enol acetate.



¹H NMR analysis of the product showed that this isomer was formed exclusively.

Initial biotransformations on this compound were hydrolyses to the ketone, catalysed by pig liver esterase. This reaction comprises an initial hydrolysis followed by protonation to give the ketone. Because the substrate is achiral the resolution relies upon the protonation step occurring before the compound leaves the active site after hydrolysis. If this is the case protonation should occur from one side only and a high yield of optically active material should be achieved.

Initial experiments were performed in aqueous solution monitored by pH stat and were worked up by extraction. Problems were encountered in the work-up procedure because 2-

methylcyclohexanone appears to be exceptionally volatile and was evaporated along with the solvent.

In further experiments the product was extracted into dichloromethane and the solvent was removed very briefly on the rotary evaporator with a cold water bath. This was followed by distillation, transferring to a microapparatus when the volume was reduced enough. The amount of ketone in the distillation residue was calculated from the ^1H NMR spectrum using a known amount of chloroform as an internal reference. The rotations of the final samples were found to be 0° in each case. Optically pure 2-methyl cyclohexanone has been demonstrated to have a rotation of $[\alpha]_{579}^{+17^\circ}$ using a 4.2% solution in ethanol: benzene 1:1 and $+6.5^\circ$ using a 4.5% solution in ethanol.³¹⁸ Our solution was made up to 4.5% in ethanol and so for optically pure 2-methyl cyclohexanone the measured rotation should have been 0.54. Hence it was concluded that PLE was treating the non-chiral enol acetate completely non selectively.

This compound was also hydrolysed by various lipases in a biphasic system of isooctane: water. Isooctane was chosen on the basis that it had been shown to be a useful solvent for enzymatic reactions on previous occasions and the initial experiments used an isooctane: water ratio of 2 ml: 5 ml. This was later varied from 2:4 to 2:8 with no apparent effect upon the reaction. The biphasic reaction mixtures were stirred at room temperature and reaction was followed by GLC using SE30 3% at 100°C with injector and detector temperatures of 125°C . This gave retention times for the enol acetate and ketone of 7 min and 5 min respectively. The

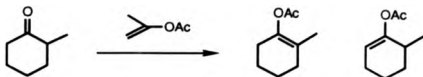
reactions with lipase from *Candida cylindracea* were found to reach completion after 24h. and were stopped by separation of the phases. A rotation measurement was made on the crude isooctane layer and it was found to have a small positive rotation; $[\alpha]_D +0.94^\circ$.

The 2-methylcyclohexanone was purified from the crude isooctane solution by preparative gas chromatography and the ketone isolated was found to be completely racemic. Reactions on this system with PPL and lipases from *Rhizopus javanicus* and *Aspergillus niger* gave incomplete conversions and no rotation of the isooctane phase.

6.2.2 EXPERIMENTS WITH THE CHIRAL ENOL ACETATE

It has been reported that in the preparation of enol acetates of unsymmetrical ketones, heating with acetic anhydride affords the more highly substituted enol acetate while use of isopropenyl acetate gives large amounts of the less highly substituted isomer.³¹⁹ However, heating 2-methylcyclohexanone with isopropenyl acetate in the presence of a *p*-toluene sulphonic acid catalyst repeatedly produced a mixture of the two isomers.

Scheme 72. Preparation of the enol acetate using isopropenyl acetate.



As expected from earlier reports the two isomers were not separable on GLC.^{320,321} The composition of the mixture was chiral isomer: non chiral isomer 1.5: 1 as determined from the ¹H NMR spectra. An attempt was made to separate the two isomers by spinning band distillation but this proved to be unsuccessful as the acetate appeared to cleave under prolonged heating.

Since previous results had shown that products derived from hydrolysis of the non-chiral isomer of the enol acetate were unlikely to give optically active ketone, some enzymatic hydrolyses were performed on the mixture of isomers. The reactions were again performed in a biphasic mixture of isooctane and water. If the isooctane phase from these experiments was found to exhibit a rotation it could be assumed that the optically active ketone was derived from the chiral enol acetate. Results from some of these experiments are summarised below.

Reactions were performed on 90-100 mg of the substrate with 10-20 mg of the appropriate enzyme.

Specific rotations were calculated allowing corrections for the product from the non-chiral enol acetate having no rotation.

Table 20. Results of the screening experiment for hydrolysis of the enol acetate.

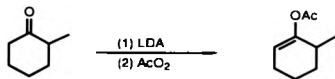
Lipase source	Rotation of crude isoocane
<i>Candida cylindracea</i> (Amano)	+ 4.63°
* <i>Pseudomonas</i> sp. (Amano)	0°
* <i>Rhizopus javanicus</i> (Biocatalysts)	0°
*Pig pancreas (Sigma)	+ 1.37°
<i>Aspergillus niger</i> (Biocatalysts)	+10.15°

*Control experiments were performed with these lipases on the non chiral isomer and although some conversion was observed in each case the rotation was always zero.

The results with lipases from *C. cylindracea* and *A. niger* proved to be reproducible on a larger scale. A further control experiment with lipase from *A. niger* in which no substrate was added showed that the optically active material was not derived from the enzyme.

Because results with the mixture of isomers seemed to be so promising the synthesis of the chiral isomer alone was attempted using another method.³²² The ketone was treated with lithium diisopropylamide followed by quenching with acetic anhydride to give the chiral enol acetate in 70% yield.

Scheme 73. Preparation of the chiral isomer.



This material was characterised using standard spectroscopic techniques.

This was then subjected to enzymatic hydrolysis with lipases from *C. cylindracea* and *A. niger* in the two phase system, terminating the reaction at 50% hydrolysis as judged by the peak areas of substrate and product on GLC. The products in each case were isolated by brief evaporation of the isooctane followed by kugelrohr distillation. The reaction with CCL gave a low yield of ketone which had a rotation $[\alpha]_D +1.6^{\circ}$ (c 3.05, CHCl_3).

The reaction with lipase from *A. niger* was unreliable as sometimes it was complete in 3h. and at others it required more than 24h. The rotation of the crude isooctane phase showed a value of $[\alpha]_D +13.4^{\circ}$ for one of the reactions but when purified the ketone had virtually no rotation. It should be noted also that kugelrohr distillation failed to separate the ketone completely from the enol acetate and so the samples were never completely pure. It was also investigated whether the ketone could be purified by flash chromatography but this proved unsuccessful.

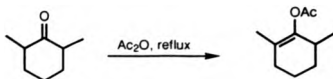
6.3 RESOLUTION OF 2,6-DIMETHYL CYCLOHEXANONE

2,6-Dimethylcyclohexanone does not have the problems associated with isomeric enol acetates as does 2-methylcyclohexanone. The enol acetate was however, prepared from commercially available ketone which is a mixture of *cis* and *trans* isomers.

Initially attempts to prepare the enol acetate were by treatment with lithium diisopropylamide followed by acetic anhydride but in

this instance this method was unsuccessful. The enol acetate was prepared by heating under reflux in acetic anhydride.³²³

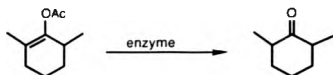
Scheme 74. Preparation of the enol acetate from 2,6-dimethylcyclohexanone



The enol acetate was identified by its ^1H NMR spectrum.

In the enzymatic hydrolysis of this enol acetate the enzyme should recognise the chirality at C-6 and either a single diastereomer or a pair can be formed in generation of the second chiral centre.

Scheme 75. Hydrolysis of the enol acetate.



Hydrolysis of this enol acetate with PLE in an aqueous system was unsuccessful. The reactions were performed on the pH stat and the uptake of alkali was either erratic or no consumption of alkali was observed at all. No product was isolated in any instance. A screening experiment was also performed on hydrolysis with lipases in the two-phase system as used previously in the hydrolysis of the enol acetate of 2-methylcyclohexanone. The reactions were each performed on 100 mg of the enol acetate and the lipases used are detailed below.

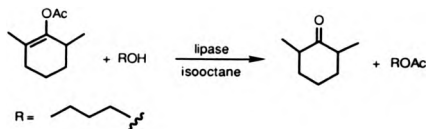
Table 21. Lipases used in the screening experiment for enantioselective hydrolysis of the enol acetate of 2,6-dimethylcyclohexanone.

Lipase source	Supplier	Amount (mg)
<i>C. cylindracea</i>	Amano	40
Pig pancreas	Sigma	40
<i>Rhizopus</i> sp.	Amano	42
<i>Mucor</i> sp.	Amano	52
<i>Pseudomonas</i> (Lipase P)	Amano	41
<i>A. niger</i>	Biocatalysts	45

The reactions were followed by GLC using SE 30 10% at 140°C or 120°C with injector and detector temperatures of 200°C, and in each case a trace of product was evident after 3 days. These reactions were apparently too slow to be of practical use and were abandoned.

A final attempt on the resolution of this ketone was made with a transesterification reaction of the enol acetate with n-pentanol.

Scheme 76. Transesterification



A screening experiment was performed with 9 lipases on 10 mg substrate in a solution of 0.1 ml pentanol and 0.9 ml isooctane. A

control experiment was performed without enzyme. The lipases used are detailed below.

Table 22. Lipases used for the transesterification reaction

Lipase source	Supplier	Amount (mg)
Pig pancreas	Sigma	21
Lipase P	Amano	17
<i>Candida cyclindracea</i>	Amano	17
<i>Aspergillus</i> sp	Amano	35
<i>Rhizopus</i> sp	Amano	24
<i>Mucor</i> sp	Amano	18
<i>Penicillium cyclopium</i>	Biocatalysts	23
<i>Geotrichum candidum</i>	Biocatalysts	21
<i>Candida lipolytica</i>	Biocatalysts	16

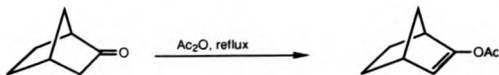
The reactions were again monitored by GLC (conditions as previously) but after 5 days no change was observed in any of the reactions. It was concluded that this substrate was too hindered to fit into the active site of the enzymes.

6.4 RESOLUTION OF NORCAMPHOR

6.4.1 SYNTHESIS OF THE ENOL ACETATE

The synthesis of the enol acetate of norcamphor was initially attempted using refluxing acetic anhydride as this method is fast and simple to apply.

Scheme 77. Preparation of the enol acetate of norcamphor.



Despite repeated attempts this synthesis was unsuccessful, leading only to the recovery of norcamphor.

The second method used involved heating the ketone with isopropenyl acetate and an acid catalyst.³²⁴ The reaction conditions were gradually made more vigorous and the composition of the reaction mixture checked by GLC (PEG 20M at 100°C , injector and detector 250°C) but no change in composition was observed. Working up the reaction yielded starting material with a trace of isopropenyl acetate.

The desired compound was finally synthesized by treatment of the ketone with lithium diisopropylamide followed by quenching with acetic anhydride.³²¹ The enol acetate was initially obtained in 3% yield after kugelrohr distillation and flash chromatography. This was later improved to 51% by not performing the distillation but using an improved flash chromatography system. The enol acetate was identified and characterised by a full range of spectroscopic techniques and the composition was confirmed by elemental analysis. The ^1H NMR spectra of compounds such as this are difficult to interpret fully because the couplings are complicated and all of the resonances fall into a small area of the spectrum. However the appearance of resonances due to the vinylic and

acetate protons and identification of the protons at C-1 and C-4 confirm that the desired enol acetate was formed.

6.4.2 ENZYME-CATALYSED RESOLUTIONS

Experiments were performed on the hydrolysis of the norbornanone enol acetate using pig liver esterase.

Scheme 78. Hydrolysis of norcamphor enol acetate.

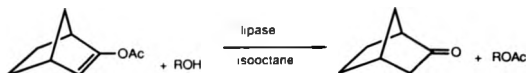


The reaction was followed by uptake of sodium hydroxide solution from the autoburette. The hydrolysis went to 100% completion with no rate change at any point on the curve. A control experiment without enzyme showed that no spontaneous hydrolysis was occurring over two days.

A hydrolytic reaction was also performed using lipase from *Candida cylindracea* in an isooctane-water two phase system. The reaction was followed by GLC of the isooctane phase on SE30 10% at 100°C with injector and detector temperatures of 200°C. The reaction was found to go to completion. If it were completely selective it would be expected to stop at 50% conversion. However in this case the rate was not studied and it may be that one enantiomer was consumed more rapidly than the other.

The speed of the reaction with PLE however suggested that the compound, although apparently stable in water was readily labile and so it was decided to attempt the resolution by an enzyme-catalysed transesterification reaction.

Scheme 79. Lipase-catalysed transesterification of norcamphor enol acetate



Initially experiments were performed with various lipases and heptanol on 50mg substrate. The reactions were monitored daily by filtering out the enzyme and measuring the rotation of the reaction mixture. There has been some variation in the reported rotation of optically pure norcamphor. It is reported to be $[\alpha]_D +28.3^\circ$ (in CHCl_3),³¹³ $[\alpha]_D + 15^\circ$ (in CHCl_3)³¹⁴ or $[\alpha]_D +17^\circ$ (in CHCl_3).³¹⁵ Although the figure varies it is a sufficiently large rotation to be measurable on a fairly dilute solution. The lipases used and the final rotations obtained are shown below (α =measured rotation, not specific rotation).

Table 23. Results of a screening experiment for the transesterification of norcamphor enol acetate.

Lipase source	Amount (mg)	Rotation
Pig pancreas (Sigma)	30	+0.026°
<i>C. cylindracea</i> (Amano)	12.8	+0.058°
Lipase P (Amano)	24	0
<i>A. niger</i> (Biocatalysts)	33	+0.021°
<i>Mucor</i> sp (Amano)	17	+0.035°
Control (No lipase)		+0.034°

The figure obtained for the control shows that none of these rotations are significant. Heptanol was chosen for the initial experiment because it has been demonstrated that lipases are highly active upon this alcohol.²⁹⁶ However in this instance it has the disadvantage that it has an identical retention time to norcamphor on GLC. Hence further transesterification experiments were performed using octanol and pentanol and were followed by GLC. The results of a screening experiment are shown below:

Table 24. Results of further screening work on the transesterification of norcamphor enol acetate.

Lipase source	Conversion after 3 days.	
	Pentanol	Octanol
Pig pancreas	No reaction	Trace of product
<i>C. cylindracea</i>	Approx 50% conv.	Approx 50% conv.
Lipase P.	Small amount product.	Small amount product
<i>Rhizopus javanicus</i>	Approx 50% conv.	No reaction.
<i>Aspergillus niger</i>	Approx 50% conv.	No reaction
<i>Mucor</i> sp.	Trace of product	No reaction.

Various attempts were made to scale up the reaction and isolate the optically active ketone by preparative GLC or preparative TLC without success. Due to the high volatility of these compounds trapping from preparative GLC was difficult as was their observation on preparative TLC.

Finally preparative scale (100 mg substrate) experiments were performed with various enzymes selected from the screening work. The remaining enol acetate from the hydrolysis was isolated by flash chromatography, hydrolysed by methanolic HCl and the resultant norcamphor was converted into its 2,4-dinitrophenylhydrazone derivative. The specific rotation of the DNP-derivative of optically pure norcamphor has been reported to be $[\alpha]_D -51.3^\circ$ (c 2.8%, CHCl_3).³¹¹

In experiments with pentanol and lipases from *A. niger* and *R. javanicus* little conversion was observed in the preparative scale work. In the cases of lipase from *C. cylindracea* and lipase P samples of DNP-norcamphor were obtained with specific rotations of 0° and $+5.33^\circ$ respectively. For lipase P this represents an enantiomeric excess of 10.3%. No attempts have been made to optimise the lipase P catalysed reaction.

6.5 SUMMARY AND CONCLUSIONS

The enol acetates of the three ketones were synthesised and were successfully hydrolysed using PLE and some lipases. In the case of the 2-methylcyclohexanone example no enantioface discrimination

was observed in hydrolysis of the non-chiral isomer of the enol acetate and although initially results with the chiral isomer appeared promising, no optically active ketone was isolated. In the case of 2,6-dimethylcyclohexanone little reaction was observed with any of the lipases - this was a totally unexpected outcome.

In the case of norcamphor enol acetate the transesterification with n-pentanol catalysed by lipase P gave a small degree of enantioselectivity. It is feasible that optimisation of the conditions could improve this.

However despite literature precedence in the resolution of enol acetates via hydrolysis of their enol acetates using microorganisms a similar result appeared to be unobtainable using commercially available lipases.

CHAPTER 7

EXPERIMENTAL DETAILS

7.1 INTRODUCTION

All chemicals and solvents were purified according to literature procedures.³²⁵

Proton nuclear magnetic resonance spectra were recorded using a Bruker WH 400, operating at 400.13 MHz or a Perkin Elmer R34 operating at 220 MHz. ¹³C Nuclear magnetic resonance spectra were recorded on a Bruker WH 400 operating at 100.62 MHz. Chemical shifts are quoted in ppm downfield from a tetramethylsilane internal reference. Mass spectra were recorded using a Kratos MS 80 spectrometer. Infra red spectra were recorded using a Perkin Elmer 580B instrument, and either a liquid film or Nujol Mull on sodium chloride plates, or on a 5% solution.

HPLC was performed on a Waters Associates instrument using an Anachem Spherisorb C-18 reverse phase column.

Optical rotations were measured on an "Optical Activity Ltd" AA 1000 polarimeter at 589 nm in a 2 dm path length cell.

Enzyme hydrolyses were performed on a Radiometer Ltd recording pH stat comprising PHM 82 pH meter, TIT 80 titrator, ABU 80 autoburette and TTA 80 titration assembly.

Gas chromatography was performed on a Pye Unicam 204 instrument using 1.8 meter long, packed columns. Nitrogen was used as the carrier gas with a flow rate of 30 ml/min.

Thin layer chromatography was performed using Merck kieselgel F254 0.2mm precoated aluminium plates. Visualisation was by one or more of the following methods:

- UV fluorescence quenching
- exposure to iodine vapour
- 5% potassium permanganate in water followed by gentle heating
- 10% phosphomolybdic acid in ethanol followed by heating to 150°C
- methyl red (0.02 g) in ethanol (60 ml) and water (40 ml) (acids).

Melting points were determined using a Gallenkamp apparatus and are quoted uncorrected.

Flash chromatography was performed on Merck Kieselgel 60 silica gel (230-400mesh).

Solvents were removed using a Büchi rotary evaporator with a maximum water bath temperature of 45°C

Ether refers to diethyl ether and petrol to the petroleum fraction boiling in the range 40-60°C.

7.2 EXPERIMENTAL TO CHAPTER 2.

7.2.1 Preparation of 3-[4-[2-hydroxyethyl]phenoxy]-1,2-epoxy propane.

4-Hydroxyphenylethyl alcohol (0.5 g, 3.6 mmol) was dissolved in butanone (8 ml). Potassium carbonate (0.65 g, 4.7 mmol) was added and the mixture was stirred. Epibromohydrin (0.5 g, 3.6 mmol) was added and the solution was heated under reflux for 8-18 h. until analysis by GLC showed that the reaction was complete.

The reaction mixture was cooled and filtered and the solvent was evaporated. The residue was dissolved in dichloromethane and the solution was washed thoroughly with water. The organic solution was dried (MgSO_4) and evaporated. The whitish oil which remained crystallised upon standing and the product was recrystallised from ethyl acetate/petrol.

Yield 47% (34% of the starting material was also recovered)

Melting point $> 300^\circ\text{C}$

^1H NMR (400MHz, CDCl_3): δ_{H} 7.12 (2 H, d, J 9 Hz, Ar-H), 6.85 (2 H d, J 9 Hz, Ar-H), 4.16-4.20 (1 H, dd, J 3, 11 Hz, O- CH_2 -epox), 3.89-3.93 (1 H, dd, J 6, 11 Hz, O- CH_2 -epoxide), 3.76-3.80 (2 H, t, J 6.6 Hz, HO- CH_2 -), 3.34-3.31 (1 H, m, CH-epoxide), 2.87-2.89 (1 H, t, J 4.5 Hz, CH_2 -epoxide), 2.76-2.79 (2H, t, J 6.5Hz, HOCH_2CH_2), 2.72-2.74 (1 H, dd, J 2.5, 5 Hz, CH_2 -epoxide).

^{13}C NMR (CDCl_3): δ_{C} 157.0 (aromatic C-O), 131.0 (CH_2 -C_{aromatic}), 129.8 (2C-aromatic), 127.0 (2C-aromatic), 68.7 (O- CH_2 -epox), 63.6 (HO- CH_2 -), 50.0, 44.5 (epoxide C), 38.1 (HO- CH_2 - CH_2 -)

IR (Nujol Mull): ν 3100-3400br, s, 1565m, 1065m, cm^{-1} .

Mass spectrum (+EI): m/z 194 (M)⁺, 163 (97.0), 107 (100.0), 77 (22.7), 31 (39.4)

Elemental analysis: Found C, 67.8; H, 7.3%. $\text{C}_{11}\text{H}_{14}\text{O}_3$ requires C, 68.0; H, 7.27%.

7.2.2 Preparation of 3-[4-[2-acetoxyethyl]phenoxy]-1,2-epoxypropane.

3-[4-[2-Hydroxyethyl]phenoxy]-1,2-epoxypropane (1 g, 5.15 mmol) was dissolved in dry tetrahydrofuran (10 ml) in a flask equipped with seal and stirrer bar, and the solution was cooled to 0°C. Triethylamine (0.52 g, 5.15 mmol) was added and the mixture was stirred for 10 min. Acetylchloride (0.4 g, 5.15 mmol) was added and stirring was continued. The mixture was allowed to warm up to room temperature over several hours and the reaction was followed by TLC (eluting with ether: toluene 1:1). After the disappearance of all of the starting material the solution was filtered and diluted with dichloromethane (20-30 ml). The organic solution was washed with 1M aqueous hydrochloric acid (~20 ml), saturated aqueous sodium hydrogen carbonate solution (~20 ml) and water (20 ml). The solution was then dried over magnesium sulphate and evaporated. The product was purified by flash chromatography, eluting with ether: hexane 1:2 to give the product as a clear oil.

Yield 34%.

¹H NMR (400MHz, CDCl₃): δ_H 7.15 (2 H, d, *J* 9Hz, Ar-H), 6.84 (2 H, d, *J* 9Hz, Ar-H), 4.22 (t, *J* 7Hz) 4.17 (dd *J* 3, 11Hz) (3H, AcO-CH₂ and O-CH₂-epoxide), 3.91 (1 H, dd, *J* 6, 11 Hz, OCH₂-epoxide), 3.32 (1 H, m, CH-epoxide), 2.86 (3 H, m, AcOCH₂CH₂), and 1 H of

CH₂ of epoxide), 2.73 (1 H, dd, *J* 2.7, 5 Hz, H of CH₂ of epoxide),
2.01 (3 H, s, COCH₃).

¹³C NMR (CDCl₃): δ_C 170.8 (CH₃C=O), 157.1 (Ar-C=O), 130.3 (-CH₂-C-Ar), 129.7 (2C-Ar), 114.6 (2C-Ar), 68.7 (Ar-O-CH₂), 64.9 (Ac-O-CH₂), 49.9, 44.5 (epoxide), 34.1 (AcO-CH₂-CH₂-Ar), 20.8 (CH₃CO-).

IR (neat liquid): ν 2900, 3000 br, w, 1740s, 1620w, 1040m, cm⁻¹.

Mass spectrum (+EI): *m/z* 236 (M)⁺, 176 (64.8), 163 (20.4) 133 (38.2), 120 (55.3), 107 (50.7).

Elemental analysis: Found C, 65.87; H, 6.82%. C₁₃H₁₆O₄ requires C 66.1; H 6.85%.

7.2.3 Preparation of 1-chloro-2-hydroxy-3-[4-(2-acetoxyethyl)phenoxy]propane.

3-[4-[2-Acetoxyethyl]phenoxy]-1,2-epoxypropane (6 g) was dissolved in dry dichloromethane. The mixture was stirred at room temperature and dry hydrogen chloride gas was bubbled in. The reaction was followed by TLC, eluting with ether: toluene 1:1 and was complete after 30 min. The gas flow was stopped and the mixture was diluted with dichloromethane. The solvent was evaporated. The product was purified by flash chromatography, eluting with ethyl acetate: petrol 1:1, and was obtained as a colourless oil.

Yield 59%.

¹H NMR (400MHz, CDCl₃): δ_H 7.12 (2 H, d, *J* 8.6 Hz, Ar-H), 6.84 (2 H, d, *J* 8.6 Hz, Ar-H), 4.20 (3 H, t, *J* 7 Hz, m, AcOCH₂, and HO-CH),

4.05 (2 H, dd, J 3, 5.6 Hz, $O-CH_2$ $CHOH-$), 3.68-3.78 (2 H, qd, J 5.2, 11 Hz, CH_2Cl), 2.85 (2 H, t, J 7 Hz, $AcO-CH_2-CH_2-Ar$), 2.02 (3 H, s, CH_3CO).

^{13}C NMR ($CDCl_3$): δ_C 170.86 (CH_3CO), 156.9 ($Ar-C-O$), 130.6 (CH_2-C-Ar), 129.8 (2C-Ar), 114.6 (2C-Ar), 69.8 ($-CHOH-$), 68.6 ($ArOCH_2-$), 64.9 ($AcOCH_2$), 45.8 ($-CH_2Cl$), 34.1 ($AcOCH_2CH_2-$), 20.8 (CH_3CO-)

Mass spectrum (+CI): m/z 272 (M^+), 254 (21.6), 212 (79.1), 177 (19.2), 163 (12.5), 120 (64.8), 107 (40.9).

IR (neat liquid): ν 3450br, 2960w, 2900w, 1740s, 1732s, 1615m, 1515s, 1240s, 1035m, cm^{-1} .

Elemental analysis: Found C, 57.73; H, 6.39; Cl, 11.85%. $C_{13}H_{16}O_4Cl$ requires C, 57.25; H, 6.3; Cl, 13.0%.

7.2.4 Preparation of 1-chloro-2-butanoyloxy-3-[4-[2-acetoxyethyl]phenoxy]propane

The α -chlorohydrin (1.0 g, 3.6 mmol) was dissolved in pyridine (5 ml), and dichloromethane (10 ml). N,N -dimethylaminopyridine (0.2 g) was added and the mixture was stirred. Butyric anhydride (0.725 g, 4.58 mmol) was added, the flask was stirred at room temperature and reaction was followed by TLC, eluting with ether: toluene 1:1. When the reaction was complete (disappearance of the starting material was observed after 60 min) the solution was diluted with dichloromethane (50 ml). The resultant solution was washed with 1M aqueous hydrochloric acid (50 ml), 1M aqueous sodium carbonate solution (50 ml) and water (50 ml). The organic

solution was then dried (MgSO_4) and evaporated. The product was purified by flash chromatography, eluting with petrol : ethyl acetate 7:3, and was obtained as a colourless oil.

Yield 70%.

^1H NMR (400 MHz, CDCl_3): δ_{H} 7.12 (2 H, d, J 9 Hz, Ar-H), 6.84 (2 H, d, J 9 Hz, Ar-H), 5.30-5.36 (1 H, quin, J 5.1 Hz, $\text{CH}(\text{OBu})$), 4.23 (2 H, t, J 7 Hz, AcO-CH_2), 4.13 (2 H, dd, J 2, 5 Hz, Ar-O- CH_2 - $\text{CH}(\text{OBu})$), 3.74-3.86 (2 H, qd, J 5, 14 Hz, CH_2Cl), 2.86 (2 H, t, J 7 Hz, $\text{AcOCH}_2\text{CH}_2$ -), 2.32-2.36 (2 H, td, J 2, 7 Hz, C-2'), 2.02 (3 H, s, $\text{CH}_3\text{CO-}$), 1.64-1.69 (2 H, q, J 7.4 Hz, C-3'), 0.93-0.96 (3 H, t, J 7.4 Hz, C-4').

^{13}C NMR (CDCl_3): δ_{C} 172.4 (C-1'), 170.5 ($\text{CH}_3\text{C=O}$), 156.8 (Ar-C-O), 130.5 (CH_2 -C-Ar), 129.6 (2C-Ar), 114.5 (2C-Ar), 70.6 (CHOBu), 66.0 ($\text{ArOCH}_2\text{-CHOBu}$), 64.7 (AcOCH_2), 42.3 (- CH_2Cl), 35.8 (C-2') 33.9 ($\text{AcOCH}_2\text{CH}_2$ -Ar), 20.6 ($\text{CH}_3\text{-CO-CH}_2\text{CH}_2\text{Ar}$), 18.1 (C-3'), 13.2 (C-4').

Mass spectrum (+EI): m/z 343 (M^+), 281 (8.3), 163 (100), 133 (5.9), 120 (12.6), 107 (12.2).

IR (neat liquid): ν 2960s, 2940m, 2800m, 1740s, 1615s, 1580m, 1515s, 1460m, cm^{-1} .

Elemental analysis: Found C, 59.65; H, 6.81; Cl, 10.42%. $\text{C}_{17}\text{H}_{23}\text{O}_4\text{Cl}$ requires C, 59.56; H, 6.76; Cl, 10.34%.

7.2.5 Preparation of the Mosher's ester

The α -chlorohydrin product (20 mg) and (S)-(+)-MTPA-chloride (50 mg) were placed in a dry flask under a positive pressure of nitrogen. Pyridine (2 drops ~0.1 ml) was added followed by dichloromethane (1 ml). The solution was stirred at room temperature and reaction was followed by TLC, eluting with toluene: ether 1:1. When all of the starting material had disappeared (usually after 1.5-2 h.) the reaction was worked up. Water (10 ml) and 1M aqueous hydrochloric acid (30 ml) were added and the solution was stirred. The product was extracted into dichloromethane (2 x 50 ml) and the combined organic extracts were washed with saturated aqueous sodium carbonate solution (50 ml) and water (50 ml). The solution was dried (MgSO_4) and evaporated.

The product was purified by preparative TLC, eluting with ethyl acetate: petrol 1:1.

Mosher's ester (of the racemic α -chlorohydrin).

Yield 80%.

^1H NMR (400 MHz, CDCl_3): δ_{H} 7.55 (2 H, m, MTPA-Ar-H), 7.35 (3 H, m, MTPA-Ar-H), 7.13 (1 H, d, J 9 Hz, Ar-H), 7.10 (1 H, d, J 9 Hz, Ar-H), 6.82 (1 H, d, J 9 Hz, Ar-H), 6.75 (1 H, d, J 8 Hz, Ar-H), 5.6 (1 H, m, CH-O-MTPA), 4.23 (3 H, m, AcOCH_2 and 1 H of Ar-O-CH_2), 4.13 (1 H dd, J 2 Hz, 1H of ArOCH_2), 3.75-3.9 (2 H, m, CH_2Cl), 3.59, 3.55 (3 H, 2 x complex s, OMe of the two diastereoisomers), 2.84 (2 H, complex q, J 7 Hz, $\text{AcOCH}_2\text{CH}_2$), 2.02 (3 H, s, CH_3COO).

7.2.6 General procedure for the enzymatic hydrolysis of the butanoate ester

A solution of the ester (500 mg), acetone (3.5 ml), 0.06M phosphate buffer, pH 7 (31.5 ml) and the microbial lipase (250 mg) was stirred at room temperature. Aqueous sodium hydroxide solution (0.1M) was added via a pH-stat to maintain a pH of 7.0. After addition of the theoretical amount of alkali the reaction mixture was acidified to inactivate the enzyme. The solution was reneutralised with aqueous sodium hydroxide solution and then the solvent was evaporated. The residue was freeze-dried to remove as much of the water as possible and then was stirred with ethyl acetate to extract the product and unreacted starting material. The organic solution was filtered, and evaporated. The products were separated by flash chromatography eluting with ethyl acetate:petrol 1:3.

α -Chlorohydrin yield 56 mg.

Ester yield 219 mg.

Over repeated runs the yield of the α -chlorohydrin ranged from 32 mg to 108 mg. Yields of the ester ranged from 219 mg to 310 mg. Comparable results were obtained with both enzymes. Spectral data for the optically active ester and α -chlorohydrin were identical to those for the racemic compounds. The optically active α -chlorohydrin (10-20 mg in each case) was converted into its Mosher's ester using the method described above.

Yield 15.5 mg (from 20 mg α -chlorohydrin).

¹H NMR (400 MHz, CDCl₃): As for the racemic sample, except that the multiplets have simplified and resonances due to the α-chlorohydrin aromatic protons are no longer split into equal doublets. The ratio of the two resonances at δ_H 3.59, 3.55 ppm due to the OMe of the diastereoisomers has changed from 1:1 to 20:1, showing that the sample has 90%ee. As mentioned in Chapter 2 no attempt was made to assign the absolute configuration, although it was observed that both lipases were selective for the same enantiomer. Comparable results were obtained for the two enzymes over multiple runs.

7.3 EXPERIMENTAL TO CHAPTER 3

7.3.1 Preparation of methyl 3-oxo-4-(*p*-chlorophenylthio)-butanoate

p-Chlorothiophenol (43.0g, 0.297 mole) was dissolved in pyridine (35.24 g, 0.45 mole, 1.5 equiv) and methyl 4-chloroacetoacetate (44.72 g, 0.297 mole) was added gradually. When the addition was complete (approx 30 min.) the mixture was stirred at room temperature for two hours during which time a precipitate of pyridinium chloride formed. The reaction mixture was neutralised with hydrochloric acid (0.5 M, ~300 ml) and the product was extracted with ether. The combined organic extracts were washed with hydrochloric acid and dried over magnesium sulphate. The ether was evaporated, and the product recrystallised from ether/pentane.

Yield 41.8%.

Melting point 50.4 - 50.7°C.

^1H NMR (CDCl_3 , 220MHz): δ_{H} 7.32 (4 H, s, Ar-H), 3.82 (2 H, s, C(4)H) 3.75 (3 H, s, OCH_3), 2.68 (2 H, s, C(2)H).

IR (CHCl_3): ν 1747 s, 1717s, 1655w, 1523w, 1478m, 1320m, 1096s, 1037s, cm^{-1} .

Mass Spectrum (EI): m/z 258 (M^+), 226 (10.3), 184 (41.6), 157 (100.0).

The data for this compound were as described previously.²⁷⁴

7.3.2 Reduction of methyl-3-oxo-4-(*p*-chlorophenylthio)-butanoate

Methyl-3-oxo-4-(*p*-chlorophenylthio)butanoate (10 g) was dissolved in dry tetrahydrofuran (300 ml) and the solution was cooled to -20°C. Sodium borohydride was added and the mixture was stirred at -20°C. Reaction was followed by TLC, eluting with ethyl acetate: petrol 1:1.

On completion of the reaction hydrochloric acid (1M) was added until bubbling ceased. The product was extracted into ether and the combined extracts were dried over magnesium sulphate. The ether was evaporated. The product was purified by flash chromatography, eluting with ethyl acetate/petrol 1:3 followed by recrystallisation from ether/pentane.

Yield 76%.

Melting point 40.5-41.7°C.

^1H NMR (220MHz, CDCl_3): δ_{H} 7.35 (4 H, m, H aromatic), 4.2 (1 H, m, C(3)H), 3.75 (3 H, s, OCH_3), 3.2 (1 H, d, J 4.7 Hz, OH), 3.10 (2 H, m, C(4)H), 2.65 (2 H, m, C(2)H).

Mass Spectrum (+EI): 260 (M⁺), 242 (39.2), 183(16.7), 158(38.6), 143(26.2), 103 (76.5), 75 (24.6), 71 (82.1), 61 (35.8), 43 (100).

IR (CHCl₃): ν 3550br, 3000, 2940, 2920w, 1730s, 1480s, 1440m, 1095s, 1010m cm⁻¹.

This compound has been fully characterised.²⁷⁴

7.3.3 Preparation of methyl 3-acetoxy-4-(*p*-chlorophenylthio)-butanoate.

A solution of methyl 4-(*p*-chlorophenylthio)-3-hydroxybutanoate (25 g, 0.094 mole), and acetic anhydride (33 g, 0.324 mole) in pyridine (64 g, 0.81 mole) was stirred at room temperature for approximately 2-4 hours. Progress of the reaction was followed by TLC (ethyl acetate : petrol 2:3). Upon completion of the reaction ether (100-200ml) was added. The mixture was washed with aqueous hydrochloric acid (0.5M, 2 x 100 ml) and with aqueous sodium bicarbonate solution (1M, 1 x 100 ml). The organic solution was then dried over magnesium sulphate and evaporated. The product was purified by flash chromatography (ethyl acetate: petrol 3:7) and was obtained as a clear oil.

Yield: 40%

¹H NMR (220MHz, CDCl₃): δ _H 7.4-7.3 (4 H, m, Ar-H), 5.35 (1 H, m, C(3)H), 3.7 (3 H, s, -OCH₃), 3.2 (2 H, m, C(4)H), 2.8 (2 H, dq, *J* 14.8, 4.9 Hz, C(2)H), 1.99 (3 H, s, OCOCH₃).

Mass Spectrum (+EI): 302 (M⁺), 242 (71.7), 211(7.4), 183(42.3), 108 (15.7), 99 (42.2), 71 (10.2), 55 (7.2), 43 (100).

IR (CHCl₃): ν 3000w, 2940w, 1730s, 1470m, 1430w, 1160m,
1090m, 1005m cm⁻¹.

This compound has been fully characterised.²⁷⁴

7.3.4 Preparation of methyl-3-butanoyloxy-4-(p-chlorophenylthio)-butanoate

A solution of methyl 4-(p-chlorophenylthio)-3-hydroxybutanoate (1 g, 3.84 mmol), butyric anhydride (1.82 g, 11.5 mmol), pyridine (1.52 g, 19.2 mmol) and DMAP (0.46 g) in dry dichloromethane (50 ml) was refluxed for 2 hours. The reaction mixture was cooled, washed with aqueous hydrochloric acid (1 M, 3 x 50 ml) and aqueous sodium carbonate solution (1 M, 2 x 50 ml). The organic phase was dried over magnesium sulphate and evaporated. Further purification was by flash chromatography (ethyl acetate: petrol 1:4) and the product was obtained as a clear oil.

Yield 62%.

¹H NMR (400MHz, CDCl₃) δ _H 7.3 (2 H, m, Ar-H), 7.2 (2 H, m, Ar-H), 5.3 (1 H, m, C(3)H), 3.64 (3 H, s, O-CH₃), 3.22 (1 H, dd, *J* 14.1, 5.76 Hz, C(4)H), 3.10 (1 H, dd, *J* 14.2, 6.5 Hz, C(4)H), 2.80 (1 H, dd, *J* 16.0, 5.3 Hz, C(2)H), 2.67 (1 H, dd, *J* 16.0, 7.5 Hz, C(2)H), 2.15 (2 H, m, C(2')H), 1.59 (2 H, m, C(3')H), 0.92 (3 H, t, *J* 7.4 Hz, C(4')H).

¹³C NMR (CDCl₃): δ _C 172.6, 170.2 (C-1, C-1'), 133.8, 132.6, 131.0, 129.1 (C-aromatic), 68.9 (C-3), 51.7 (OCH₃), 37.5, 37.1, 36.0 (C-2, C-4, C-2'), 18.2 (C-3'), 13.4 (C-4').

IR (neat liquid): ν 2960m, 2920, 2860w, 1760, 1740s, 1470m,
1430m, 1380w, 1300w, 1090m, 1000m, cm⁻¹.

Mass spectrum (EI): m/z 330(M^+), 299 (0.8), 242 (100.0), 211 (4.7), 183 (48.1).

This compound has been fully characterised.²⁷⁴

7.3.5 Enzymatic hydrolysis of the acetate ester with PPL.

A solution of the acetoxy compound (250 mg), PPL (100 mg) and methanol (2 ml) in 0.06M phosphate buffer pH 7.0 (18 ml) was stirred at room temperature. The pH was maintained at pH 7.0 by addition of aqueous sodium hydroxide solution *via* a pH stat. After addition of 50% of the theoretical amount of sodium hydroxide solution the mixture was acidified to pH 1.5 by addition of concentrated hydrochloric acid and stirred for five minutes to inactivate the enzyme. Concentrated aqueous sodium hydroxide solution was added to attain pH 10. The product and unreacted starting material were extracted with ether (4 x 10 ml) and the compounds were separated by flash chromatography (eluting with ethyl acetate : petrol 1:1).

(S)-Methyl 4-(*p*-chlorophenylthio)-3-hydroxybutanoate

Yield 14 mg (1.3% of theoretical).

Rotation $[\alpha]_D -3.11^\circ$ (c 0.5, CHCl₃), (Rotation of the pure enantiomer $[\alpha]_D -5^\circ$).

Spectral data are identical to those of the racemic compound.

(R)-Methyl 4-(*p*-chlorophenylthio)-3-acetoxybutanoate

Yield 82% of theoretical.

Spectral data are identical to those of the racemic compound.

7.3.6 Enzymatic hydrolysis of methyl-3-butanoyloxy-4-(p-chlorophenylthio)-butanoate.

A solution of the butanoyloxy ester (500 mg), lipase P (500 mg), Triton X-100 (25 mg) and methanol (7.5 ml) in 0.06M phosphate buffer pH 7 (30 ml) was stirred at room temperature. The pH was maintained at pH 7.0 by addition of 0.5 M aqueous sodium hydroxide solution via a pH-stat.

After addition of 50% of the theoretical amount of sodium hydroxide the enzyme was inactivated by acidification to pH 1.5 with hydrochloric acid. Aqueous sodium hydroxide solution was added to give a pH value of approximately 10.0-11.0 and the product and unreacted starting material were extracted with ether (4 x 20ml). The combined organic extracts were dried over magnesium sulphate and evaporated. The two compounds were separated by flash chromatography, eluting with ethyl acetate: petrol 1:5. The product was recrystallised to optical purity, as determined by ^1H NMR with a chiral shift reagent.

(S)-Methyl-4-(p-chlorophenylthio)-3-hydroxybutanoate

Yield 64% of the theoretical, 87%ee.

Melting point 55.5-56.5°C.

Recrystallised from dichloromethane/pentane

Yield 17%, >95%ee.

Melting point 57.7-58°C.

(R)-Methyl-3-butanoyloxy-4-(p-chlorophenylthio)-3-butanoate

Yield 89% of the theoretical.

Spectral data were identical to those reported for the racemic compounds.

7.3.7 Hydrolysis of unreacted methyl-3-butyroxy-4-(p-chlorophenylthio) butanoate from 7.3.6

A solution of the enantiomerically enriched ester (50 mg), PPL (100 mg), lipase P (20 mg), methanol (0.75 ml) in phosphate buffer (4.25 ml) was stirred vigorously at 40°C. Reaction was followed by TLC, eluting with ethyl acetate: petrol 1:1 until complete disappearance of the ester was observed. The product was then extracted with ether (4 x 10 ml) and the combined extracts were dried and evaporated. The product was purified by flash chromatography, eluting with ethyl acetate: petrol 1:1. (R)-methyl-3-hydroxy-4-(p-chlorophenylthio) butanoate.

Yield: 11% from the ester, 52%ee

Spectral data were identical to those obtained for the racemic compound.

7.3.8 Transesterification of methyl-3-hydroxy-4-(p-chlorophenylthio)butanoate.

Methyl-3-hydroxy-4-(p-chlorophenylthio)butanoate (500 mg) and vinyl acetate (496 mg, 3 equivalents) were dissolved in *tert*-butyl-methyl-ether (10 ml) and lipase P (240 mg) was added. The solution was stirred at room temperature and reaction was followed by TLC, eluting with ethyl acetate : petrol 1:1.

When the mixture was observed to contain approximately equal amounts of the alcohol and the ester the reaction was stopped by removal of the enzyme by filtration through a glass sinter. The solvent was evaporated and the two compounds were separated by flash chromatography.

The optical purity of the recovered alcohol was assessed directly by ^1H NMR in the presence of the chiral shift reagent as described previously. A sample of the product acetate was hydrolysed to the alcohol as described in section 7.3.7, and the enantiomeric purity was determined by ^1H NMR.

Recovered alcohol.

Yield 252 mg, 62.5%ee.

Melting point 50.6-51.7°C

Product acetate.

Yield 167 mg, 75%ee.

The recovered alcohol was recrystallised twice from dichloromethane/pentane.

Yield 14 mg, >95%ee.

Melting point 56.5-57.5°C.

7.4 EXPERIMENTAL TO CHAPTER 4.

3-Methyl-4-oxo-4(4-aminobenzyl)butanoic acid was kindly provided by SmithKline Beecham p.l.c., and has been fully characterised.

7.4.1 Preparation of methyl-3-methyl-4-oxo-4(4-aminobenzyl)butanoate

3-Methyl-4-oxo-4(4-aminobenzyl) butanoic acid (10.18 g, 49 mmol) was dissolved in methanol (100 ml, dried from magnesium). Concentrated sulphuric acid (2 ml) was added and the mixture was stirred at room temperature overnight.

The methanol was removed, the residue was dissolved in water and the pH brought to neutral by the addition of sodium carbonate. The product was extracted with dichloromethane (3 x 50 ml). The combined organic extracts were washed with water (100 ml), dried over magnesium sulphate and evaporated. The crude product was recrystallised from methanol : water to yield pale yellow crystals.

Yield 79%.

Melting point 119-120°C (lit 103.5-104.5°C for the pure enantiomers).²⁹⁷

¹H NMR (220MHz): δ_{H} 7.81 (2 H, d, J 7 Hz, Ar-H), 6.62 (2 H, d, J 7 Hz, Ar-H), 4.0 (2 H, br s, NH₂), 3.82 (1 H, q, J 7 Hz, C(3)H), 3.6 (3 H, s, CH₃OCO), 2.9 (1 H, dd, J 7, 18 Hz, C(2)H), 2.4 (1 H, dd, J 5.6, 16.3 Hz, C(2)H), 1.2 (3 H, d, J 7 Hz, CH₃).

7.4.2 Hydrolysis of methyl 3-methyl-4-oxo-4(4-aminobenzyl) butanoate with PLE (acetone or methanol cosolvent)

The ester (205 mg, 0.93 mmole) was dissolved in acetone or methanol (4 ml) and phosphate buffer (0.066 M, pH 7.1, 36 ml) was added. This mixture was equilibrated at 35-40°C and pH 7.0 with vigorous stirring and then PLE (40 μ l, 52 U) was added. The course of the reaction was followed by consumption of 0.1 M sodium hydroxide solution from an autoburette.

The reaction was stopped by acidification after consumption of 75% of the theoretical amount of sodium hydroxide solution. The solution was reneutralised and the unreacted ester was extracted with ethyl acetate at pH 8.

The combined organic solution was dried over magnesium sulphate and evaporated to leave the ester as a yellow gum which solidified upon standing. This was recrystallised from methanol: water.

Yield 19%.

Melting point 119°C.

Optical rotation $[\alpha]_D$ -0.2° (c 1, MeOH).

Spectral data were as described previously.

7.4.3 Hydrolysis of methyl 3-(4-aminobenzyl) butanoate with PLE (25% DMSO cosolvent)

The ester (500 mg, 2.26 mmol) was dissolved in DMSO (17.5 ml) and 0.2M Tris-HCl buffer (52.5 ml, pH 7.6) was added. The reaction mixture was stirred vigorously and equilibrated at 30-35°C for 30 min. The PLE (150 μ l, 195 U) was added and the reaction monitored by uptake of 100 mM NaOH solution from an

autoburette. The reaction was stopped after consumption of 50% of the theoretical amount of sodium hydroxide solution, by acidifying to pH 1.5 with concentrated aqueous hydrochloric acid. The solution was then reneutralised by addition of concentrated sodium hydroxide solution. The solution was extracted with dichloromethane at pH 8 to obtain the unreacted ester. The ethyl acetate was washed with dilute aqueous hydrochloric acid and with water, dried and evaporated. The remaining reaction mixture was reacidified and the acid product was extracted with ethyl acetate at pH 3. The combined extracts were washed with water, dried over magnesium sulphate and evaporated. The unreacted ester was purified by countercurrent extraction. The extracted material was dissolved in dichloromethane (2 ml) which was extracted with water (2 ml) ten times. The water extracts were passed sequentially down a line of test tubes each containing dichloromethane (2 ml) and shaken and re-separated from each.

Analysis of the resultant dichloromethane layers showed that the ester remained in decreasing amounts in tubes 1-4 and the DMSO and other impurities were found in the aqueous layer in tubes 8-9. The appropriate dichloromethane layers were combined, dried and evaporated. The isolated product was recrystallised from methanol/water.

The acid product was purified in a similar manner by dissolving the product in ethyl acetate (2 ml) and moving this layer sequentially along a line of ten tubes, each containing water (2 ml)

as described above. The acid was recovered from tubes 7-10, after drying and evaporating the ethyl acetate layers.

Ester.

Yield 50% of the theoretical.

Melting point 117-117.2°C

Optical rotation $[\alpha]_D +0.8^\circ$ (c 1.5% in CHCl_3).

Acid.

Yield 32% of the theoretical.

Optical rotation $[\alpha]_D +2.3^\circ$ (c 1% in MeOH).

Spectral data for both compounds were as reported previously.

7.4.4 Hydrolysis of methyl 3-methyl-4-oxo-4(4-aminobenzyl)butanoate with lipases in a two phase system.

The ester (50 mg) was dissolved in chloroform (2 ml) and this solution was added to a solution of the lipase (25 mg) in phosphate buffer (0.066M, pH 7, 2 ml). The mixture was stirred at room temperature and the reaction was monitored by TLC, eluting with ethyl acetate: methanol: ammonia 5:1:1 (R_f ester 0.9). No reaction was observed in any instance over a period of two weeks.

7.4.5 Immobilisation of PLE on sepharose.

Sephacrose 4B (aqueous suspension of 4-190 μm beads, 1 ml) was placed into a glass sinter funnel, and washed thoroughly with water followed by phosphate buffer (pH 8). The sepharose was then dried thoroughly under suction, removed from the filter and cut into very small pieces. It was placed in a small flask with PLE suspension (770 μl , 1000 U) and allowed to stand until all of the

enzyme had been absorbed. The preparation was filtered to remove excess buffer and stored at 4°C.

7.4.6 Transesterification of methyl 3-methyl-4-oxo-4(4-aminobenzoyl) butanoate with immobilised PLE or lipases.

The ester (20 mg) was dissolved in n-heptanol (2 ml) and immobilised PLE (100 U) or a lipase (10 mg) was added. The resultant mixture was stirred at room temperature and analysed periodically by TLC, eluting with ethyl acetate : petrol 3:1 containing 1% ammonia (Rf methyl ester 0.48). No change was observed in any of the reaction mixtures over a period of two weeks.

7.4.7 Reduction with bakers' yeast.

Fresh bakers' yeast (23 g) was added to a solution of sucrose (23 g) in water (150 ml) and stirred at 30°C until fermentation began (approx 30 min). The substrate (200 mg) was then added, dissolved in a minimum amount of ethanol (2.5 ml). The mixture was stirred at 30-35°C for 24h.

Celite was added to the flask and it was stirred for a further hour. The mixture was then filtered through more celite and the filter cake was washed thoroughly with ethyl acetate. The filtrate was extracted exhaustively with ethyl acetate and the combined extracts were dried over magnesium sulphate and evaporated. The product was purified by flash chromatography, eluting with ethyl acetate : methanol : ammonia 5:1:1

Yield 179mg.

^1H NMR (220MHz, CDCl_3): δ_{H} 8.58 (1 H, br s, NH), 7.95 (2 H, d, J 7 Hz, Ar-H), 7.68 (2 H, d, J 7 Hz, Ar-H), 3.92 (1 H, q, J 7 Hz C(3)H), 3.65 (3 H, s, CH_3OCO), 2.98 (1 H, dd, J 7, 14 Hz, C(2)H), 2.48 (1 H, dd, J 5, 16 Hz, C(2)H), 2.2 (3 H, s, $\text{CH}_3\text{CONH-}$), 1.21 (3 H, d, J 6 Hz, C(3)Me).

The product was racemic as shown by ^1H NMR using 2 equivalents of 2,2,2-trifluoro-1-(9-anthryl)ethanol.

7.4.8 Growth of and reduction with *Candida guilliermondii*.

2 x 25 ml portions of Difco YM broth were prepared according to the manufacturers instructions and sterilised for 20 min in a pressure cooker. The yeast cells were transferred to these bottles 12h. later from previously prepared Agar plates stored at 4°C . The jars were incubated at 30°C .

After 24h growth the solutions were turbid and 5ml aliquots were transferred from one bottle into four 100ml portions of the same broth, sterilized and cooled as previously. The flasks were shaken at $30\text{--}35^\circ\text{C}$ for 2 days. The substrate (230 mg) was dissolved in ethanol (4 ml) and 1 ml of this solution was transferred to each of the flasks of yeast prepared above and the flasks were incubated for 7-9h. Celite was then added and the solutions were stirred for 1h before being filtered through more Celite. The Celite filter was washed with dichloromethane. The filtrate was extracted with dichloromethane. Intense, stable emulsions formed which were dispersed by repeated filtration through Celite. The combined organics were washed with water, dried over magnesium sulphate

and evaporated. The products obtained were purified by drip column chromatography, eluting with ethyl acetate: methanol: ammonia 5:1:1.

Yield (from one culture flask only) 35mg.

^1H NMR (220MHz, CDCl_3): As for bakers' yeast reduction.

This compound was racemic as shown by ^1H NMR in the presence of 2,2,-trifluoro-1-(9-anthryl)ethanol.

7.4.9 Investigation of the lability of the product under conditions of yeast fermentation

An anion ion exchange resin was prepared in its OD^- form by stirring Biorad XAD-8 resin (1 g) with D_2O (2 ml) for 30min and then filtering and drying on a sinter funnel. The authentic racemic product expected from yeast reduction (165 mg) was then stirred in d_1 -methanol (3 ml) with the deuterated resin (200 mg) at room temperature. The deuterium incorporation could be assessed by ^1H NMR and was complete after 48h. The resin was filtered off and washed with a little more d_1 -methanol. The solvent was then evaporated and the compound (85 mg) recovered.

The recovered material was subjected to an incubation with fermenting bakers' yeast (10 g) exactly as described previously and was reisolated after 24h. The compound was extracted, purified by flash chromatography and recrystallised and was confirmed by ^1H NMR to still contain deuterium.

Deuterated material.

^1H NMR (220MHz, CDCl_3): δ_{H} 7.95 (2 H, d, J 7 Hz, Ar-H), 7.7 (2 H, d, J 7 Hz, Ar-H), 3.65 (s, 3 H, s, CH_3OCO), 3.0 (1 H, m, C(2)H), 2.5 (1 H, m, C(2)H), 2.2 (3 H, s, $\text{CH}_3\text{CO}-$), 1.22 (3 H, s, C(3)Me).

7.5 EXPERIMENTAL TO CHAPTER 5

7.5.1 Preparation of acetone oxime

Hydroxylamine hydrochloride (25 g) was dissolved in water (150 ml) and a slight excess of sodium hydrogen carbonate (10% aqueous solution, 150 ml), was added. Acetone (10 g, 0.172 mole) was added and the mixture was heated for 60 min. on a water bath and cooled. The oxime was extracted into ether (4 x 100 ml). The combined organic extracts were dried over magnesium sulphate and evaporated to give the oxime as colourless crystals.

Yield 76%.

Melting point 60.0-60.5°C (lit. 59°C).³⁰⁰

^1H NMR (220MHz, CDCl_3): δ_{H} 8.9 (1 H, s, OH), 1.9 (6 H, s, CH_3 , CH_3).

^{13}C NMR (CDCl_3): δ_{C} 155.19 (C-2), 21.4, 14.6 (CH_3)

Mass spectrum (EI): m/z 73(M^+), 58 (18.6), 56 (3.7), 42 (11.9).

IR (CHCl_3): ν 3580w, 3250br, 3000w, 2900w, 1670w, 1360m,

1060m, 940m cm^{-1} .

7.5.2 General method for the preparation of oximes of water insoluble ketones

Hydroxylamine hydrochloride (25 g) was dissolved in water (100 ml) and 1M aqueous sodium hydroxide (200 ml) was added. The ketone (10 g) was added, together with sufficient ethanol to generate a clear solution. The mixture was heated to 90-100°C for 2h. and then cooled in ice. If crystals did not form the product was extracted into ether (3 x 100 ml) and the combined extracts were dried over magnesium sulphate and evaporated. The oximes were purified by recrystallisation except in the case of norcamphor oxime which was distilled bulb to bulb.

2-methylcyclohexanone oxime

Recrystallised from hexane at dry ice temperature

Yield 69%.

Melting point 42-43°C (lit 42-44°C).³⁰³

¹H NMR (220MHz, CDCl₃): δ_H 3.15 (1 H, m, C(2)H), 2.35 (1 H, m, aliphatic CH), 2.0-1.3 (m, 7 H, m, aliphatic CH), 1.15 (3 H, d, *J* 6.7 Hz, CH₃).

¹³C NMR (CDCl₃): δ_C 163.1 (C-1), 37.1 (C-2), 35.5 (C-3), 25.9 (C-6), 24.6 (C-5), 23.7 (C-4), 16.7 (C-7)

Smaller peaks at 31.5, 28.2, 26.6, 26.5, 20.29, 16.1 for the minor conformer.

IR (Nujol mull): ν 3600w, 3280br, 1670w, 940s, cm⁻¹.

Mass spectrum (+EI): m/z 127 (M⁺), 110 (20.7), 95 (33.9), 69 (22.7), 55 (51.9), 41 (100.0), 39 (45.4).

2,6-Dimethylcyclohexanone oxime

Recrystallised from water at room temperature.

Yield 78%.

Melting point 67°-69°C (lit 79°C for the cis isomer).³²⁹

¹H NMR (400MHz, CDCl₃): δ_H 3.4 (1 H, m), 2.62 (1 H, m), (C(2)H, C(6)H) 1.8-1.4 (6 H, m, aliphatic CH), 1.2 (3 H, d, *J* 4.5 Hz, CH₃) 1.17 (3 H, d, *J* 4.5 Hz, CH₃).

Minor resonances at δ_H <1.16 in ¹H NMR suggest that the other conformational isomer or the ketone is a minor contaminant.

¹³C NMR (CDCl₃): δ_C 166.4 (C-1), 33.8, 31.4 (C-2, C-6), 30.7, 27.1, 20.6 (C-3, C-4, C-5), 18.1, 16.2 (C-7, C-8).

Mass spectrum (+EI): *m/z* 141(M⁺), 124 (72.3), 109 (100.0), 98 (27.2), 82 (32.6), 67 (38.1), 55 (73.6), 41 (58.9).

IR (Nujol mull): ν 2840-2960 s, 1700 s, cm⁻¹

Norcamphor oxime

Purified by Kugelrohr distillation at water pump pressure to give an oil at 140-141°C at 15mmHg or 105°C at 1 mmHg which solidified upon standing.

Yield 71%.

Melting point 50.2 - 51.6°C (formerly characterised as an oil).³³⁰

¹H NMR (400MHz, CDCl₃): δ_H 2.84 (1 H, d, C(1)H), 2.48 (1 H, m, C(4)H), 1.27-2.28 (8 H, m, ring protons).

¹³C NMR (CDCl₃): δ_C 167.7 (C-2), 42.2 (C-1), 38.9 (C-7), 35.3 (C-4), 34.6 (C-3), 27.6 (C-6), 26.9 (C-5).

Resonances due to the minor isomer at 199.3, 47.7, 38.1, 37.0, 27.2, 25.8ppm.

Mass spectrum (+EI): m/z 125 (M^+), 108 (38.3), 96 (47.4), 93 (30.0), 80 (37.8), 67 (100.0), 59 (64.7).

IR (solution in CHCl_3): ν 3240 s, br, 2960, 2860 d, s, 1695 w, 1420-1450w, 1950 s cm^{-1} .

7.5:3 General method for the preparation of oxime esters

The appropriate acid anhydride (acetic, butyric or hexanoic) (0.04 mol) was added to a solution of the oxime (0.03 mol) in absolute ether (100-200 ml). The mixture was heated under reflux for 2-3h or, in the case of norcamphor oxime esters, until all of the starting material had disappeared on TLC. The solution was poured into 1M aqueous sodium hydrogen carbonate solution (200 ml) and the product was extracted into ether (4 x 100 ml). The combined extracts were dried over magnesium sulphate and evaporated. The products were obtained as clear oils and purified by Kugelrohr distillation or flash chromatography, eluting with mixtures of acetone and toluene.

Acetone oxime acetate

Purified by Kugelrohr distillation at 15mmHg to give a colourless oil.

Boiling point 100-110°C.

Yield 83%.

^1H NMR (220MHz, CDCl_3): δ_{H} 2.15 (3 H, s, COCH_3), 2.08 (3 H, s), 2.03 (3 H, s) (CH_3 groups).

^{13}C NMR (CDCl_3): δ_{C} 168.3, 163.2 (C=N, C=O), 21.6, 19.2, 16.6 (CH_3 groups)

Mass spectrum (EI): m/z 115 (M^+), 73 (9.6), 56 (19.7), 43 (100.0).

IR (neat liquid): ν 3600br, 3500br, 2990w, 2970w, 2910w, 1750s, 1650m, 1430m, 1365s, 1270s, 1200s cm^{-1} .

This compound has been fully characterised.³⁰¹

2-Methylcyclohexanone oxime acetate

Purified by distillation using a Kugelrohr apparatus under high vacuum, to give a colourless oil.

Yield 72%.

Boiling point 90-100°C at 1.0mmHg.

^1H NMR (220MHz, CDCl_3): δ_{H} 1.3-3.5 (9 H, m, aliphatic C-H), 2.1 (3 H, s, Ac) 1.1-1.2 (3 H, d, CH_3).

^{13}C NMR (CDCl_3): δ_{C} 171.00 (C=O, Acetate), 169.11 (C-1), 36.80 (C-2), 34.76 (C-3), 25.99 (C-6), 25.27 (C-5), 23.19 (C-4), 16.71 (C-7)

Resonances due to the minor isomer: δ_{C} 31.46, 28.87, 27.87, 26.23, 19.73, 19.55, 16.33.

Mass spectrum (EI) m/z 169(M^+), 110(17.3), 95(16.5), 81(14.8), 56(3.2), 43(48.4), 18 (100.0).

IR (neat liquid): ν 2800-3000 s, 1750 s, 1660w, 1360, 1200, 940 cm^{-1} .

This compound has been fully characterised.³⁰³

2,6-Dimethylcyclohexanone oxime acetate

A colourless oil, purified by flash chromatography eluting with toluene : acetone 15:1.

Yield 69%.

^1H NMR (220MHz, CDCl_3): δ_{H} 3.4 (1 H, m), 2.9 (1 H, m) (C(2)H, C(6)H), 2.2 (3 H, s, COCH_3) 1.9-1.45 (6 H, m, aliphatic H), 1.3 (6H, overlapping doublets, CH_3 , CH_3)

^{13}C NMR (CDCl_3): δ_{C} 174.14 (Ac C=O), 168.86 (C-1), 33.63, 31.24, (C-2, C-6), 30.81, 29.07, 20.68 (C-3, C-4, C-5), 19.58, 18.44, 15.74 (C-7, C-8, CH_3CO).

Mass spectrum (CI): m/z 184 (M+H) $^+$, 166(3.9), 141(2.7), 124(100.0), 109(10.3), 95(20.7), 80(4.7), 69(2.5).

IR (CHCl_3): ν 2960, 2920, 2850m, 1760s, 1615w, 1450w, 1355w, 1200s cm^{-1} .

Elemental analysis: Found C, 65.14; H, 9.47; N, 7.15%. $\text{C}_{10}\text{H}_{17}\text{NO}_2$ requires C, 65.54; H, 9.35; N, 7.64%.

Norcamphor oxime acetate.

Purified by flash chromatography, eluting with toluene : acetone 15:1, to give a colourless oil.

Yield 73%.

^1H NMR (400MHz, CDCl_3): δ_{H} 2.99 (1 H, m), 2.45, (1 H, m) (C(1)H and C(4)H), 2.22 - 2.29 (m) and 2.13 (d) (2 H, aliphatic H), 2.03 (3 H, s, CH_3), 1.58-1.76 (2 H, m, aliphatic H), 1.37-1.47 (3 H, m, aliphatic H), 1.25-1.28 (1 H, m, aliphatic H).

^{13}C NMR (CDCl_3): δ_{C} 174.83 (C-8), 168.54 (C-2), 42.36 (C-1), 38.63 (C-7), 36.09 (C-4), 35.04 (C-3), 27.15 (C-6), 26.31 (C-5), 19.21 (C-9)

Resonances at 174.48, 40.42, 38.08, 37.21, 26.71, 25.59 due to the minor isomer.

Mass spectrum (+Cl): m/z 168 ($\text{M}+\text{H}$) $^+$, 126 (7.5), 110 (6.8), 96 (1.3), 81 (0.6), 67 (0.6), 60 (3.6), 35 (15.9), 18 (100.0).

IR (liquid film): ν 2960, 2880 cm^{-1} (m), 1760 cm^{-1} (s), 1670 cm^{-1} (w), 1190, 1220 (s, doublet) cm^{-1} .

Elemental analysis: Found C, 64.54; H, 7.79; N, 8.32%. $\text{C}_9\text{H}_{13}\text{NO}_2$ requires C, 64.65; H, 7.84; N, 8.38%.

Norcamphor oxime butyrate.

Purified by flash chromatography, eluting with ethyl acetate: petrol 1:3, to give a colourless oil.

Yield 65%.

^1H NMR (400MHz, CDCl_3): δ_{H} 3.01 (1 H, br d), 2.47 (1 H, br) (C(1)H, C(4)H), 2.29 (2 H, t, C(2')H), 2.24-2.33 (1 H, m), 2.11 (1 H, dd) (aliphatic H), 1.65 (2 H, q, C(3')H), 1.6-1.8 (2 H, m), 1.2-1.52 (4 H, m) (ring aliphatic H), 0.91 (3 H, t, C(4')H)

^{13}C NMR (CDCl_3): δ_{C} 174.91 (C-1'), 170.95 (C-2), 42.42 (C-1), 38.67 (C-7), 36.11 (C-4), 35.09 (C-3), 34.61 (C-2') 27.21 (C-6), 26.33 (C-5), 18.19 (C-3'), 13.42 (C-4')

Resonances due to the minor isomer at: δ_{C} 174.56, 40.48, 38.13, 37.31, 26.75, 25.62, 18.25.

Mass spectrum (+Cl): m/z 196 ($\text{M}+\text{H}$) $^+$, 126 (8.8), 110 (0.9), 88 (1.0), 71 (100.0), 67 (40.6), 60 (13.8), 43 (83.0).

IR (liquid film): ν 2950, 2860s, d, 1750s, 1665w, 1140s,
1080s cm^{-1} .

Elemental analysis: Found C, 67.70; H, 8.64; N, 7.21%. $\text{C}_{11}\text{H}_{17}\text{NO}_2$
requires C, 67.66; H, 8.78; N, 7.17%.

Norcamphor oxime hexanoate

A colourless oil, purified by flash chromatography, eluting
with ethyl acetate: petrol 1:3.

Yield 66%.

^1H NMR (400MHz, CDCl_3): δ_{H} 3.04 (1 H, br d), 2.49 (1 H, br) (C(1)H
and C(4)H), 2.3 (3 H, m, C(2')H, 1 ring proton), 2.13 (1 H, dd,
aliphatic ring H), 1.76 (1 H, m, aliphatic H), 1.65 (3 H, m,
C(3')H + aliphatic ring H), 1.4 - 1.55 (3 H, m, C(4')H and 1 ring
H), 1.24-1.33 (5 H, m, C(5')H and 2 ring H), 0.84 (3 H, m,
C(6')H).

^{13}C NMR (CDCl_3): δ_{C} 174.95 (C-1'), 171.22 (C-2), 42.49 (C-1), 38.74
(C-7) 36.17 (C-4), 35.15 (C-3), 32.77, 31.12 (C-2', C-3'), 27.28
(C-6) 26.40 (C-5), 24.45, 22.06, (C-4' C-5'), 13.65 (C-6')
Resonances for the minor isomer at δ_{C} 171.21, 40.54, 38.20,
37.38, 26.82, 25.70.

Mass spectrum (+CI): m/z 224 (M+H) $^+$, 126 (32.2), 116 (14.1), 110
(12.0), 99 (43.5), 67 (1.5), 58 (1.5), 43 (19.0), 18 (100.0).

IR (liquid film): ν 2950, 2870s, d, 1760s, 1670w, 1140, 1090s,d
 cm^{-1} .

Elemental analysis: Found C, 69.63; H, 9.37; N, 6.07%. $\text{C}_{13}\text{H}_{21}\text{NO}_2$
requires C, 69.92; H, 9.48; N, 6.27%.

7.5.4 General procedure for the enzymatic hydrolysis of oxime esters.

A solution of the oxime ester (100 mg) and enzyme (50-100 mg) in 0.06 M phosphate buffer pH 7 (8 ml) was stirred at room temperature. The pH was maintained constant by addition of aqueous sodium hydroxide solution (100 mM) via a pH stat. After addition of the appropriate amount of sodium hydroxide solution the mixture was acidified (hydrochloric acid), reneutralised (sodium hydroxide) and saturated with sodium chloride. The product and unreacted starting material were extracted with ether (4 x 10 ml) and the combined organics were dried (MgSO_4) and evaporated. The oxime and oxime ester were separated by flash chromatography or preparative TLC.

7.5.5 Cleavage of norcamphor oxime to the ketone.

A solution of sodium hydrogen carbonate (27 mg, 0.32 mmol) in water (1 ml) was added to a solution of norcamphor oxime (200 mg, 1.6 mmol) in dichloromethane (5 ml). The mixture was cooled to 0°C and a solution of bromine (0.1 ml, 0.25 g, 1.94 mmol) in dichloromethane (3 ml) was added dropwise with stirring. When addition was complete the icebath was removed and the mixture stirred at room temperature for 15 h.

The organic solution was separated and the aqueous phase was extracted with dichloromethane. The organic phases were combined, washed with water and saturated aqueous sodium chloride solution, dried over sodium sulphate and evaporated.

The crude product was used for preparation of the DNP derivative without further purification

Crude norcamphor

Yield: 71%

^1H NMR (220MHz, CDCl_3) δ_{H} Data as expected from Aldrich NMR Catalogue. Minor impurities at 1.3ppm, 2.4ppm, 2.95, 3.0, 3.1, 7.15-7.3 ppm)

IR (liquid film): ν 2950, 2870s, d, 1740s, 1550 cm^{-1} .

7.5.6 Preparation of the 2,4-dinitrophenylhydrazine derivative of norcamphor

Norcamphor (100 mg) was dissolved in warm methanol (5 ml) containing concentrated hydrochloric acid (0.4 ml) and 2,4-dinitrophenylhydrazine (100 mg). The solution was warmed on a steam bath for 10 min and then cooled and the precipitated solid was filtered off and washed with ethanol. The resultant red crystals were dried in a desiccator overnight before further use.

Yield: 89 mg.

Melting point: 130.5-131°C (Lit 128.5-129.5°C³²⁶, 130°C³²⁷).

^1H NMR (220MHz, CDCl_3): δ_{H} 9.2 (1 H, m, Ar-H), 8.35 (1 H, m, Ar-H), 8.0 (1 H, 2s, Ar-H), 3.05 (1 H, m, C(1)H or C(4)H), 2.77 (m, 1H, C(1)H or C(4)H), 1.4-2.9.4 (9 H, m, remaining norbornane protons and NH).

7.5.7 Large scale enzymatic hydrolysis of norcamphor oxime acetate.

Norcamphor oxime acetate (1 g) and lipase P (500 mg) were dissolved in 10mM phosphate buffer (pH 7, 50 ml) and the solution was stirred at room temperature. The pH was maintained by addition of 0.5 M sodium hydroxide solution using a pH stat. After addition of 50% of the theoretical amount of sodium hydroxide solution the reaction was stopped by acidification to inactivate the enzyme. The reaction mixture was then reneutralised by addition of sodium hydroxide solution and the unreacted starting material and product were extracted with ether (4 x 50 ml). The combined organic extracts were dried (MgSO₄) and evaporated. The product and unreacted substrate were separated by flash chromatography, eluting with toluene : acetone 15:1.

Recovered oxime acetate, yield 352 mg.

Product oxime, yield 317 mg.

Spectral data for these compounds were as anticipated.

A sample of the oxime (100 mg) was cleaved to the ketone.

Ketone yield 51.5 mg.

This was converted to its DNP derivative.

Yield of DNP-norcamphor 26 mg.

Melting point 128.3-129°C.

$[\alpha]_D + 14^\circ$ (c 1.3, CHCl₃) ($[\alpha]_D$ pure enantiomer +51°)

Upon repeating the experiment the following results were obtained.

Oxime acetate, yield 346 mg.

Oxime, yield 317 mg.

The spectral data for these compounds was as described previously.

The recovered oxime (317 mg) was cleaved to the ketone using the procedure described in section 7.5.5.

Norcamphor, Yield 416 mg.

Spectral data were as described previously.

The isolated ketone was converted to its DNP derivative as described in section 7.5.6.

Yield 427 mg.

Melting point 130.5-131°C.

The NMR spectrum was as previously.

$[\alpha]_D + 8.6^\circ$ (c 1, CHCl_3) ($[\alpha]_D$ pure enantiomer + 51°)

7.5.8 Attempted enzymatic esterification of norcamphor oxime.

Norcamphor oxime (50 mg, 0.4 mmol) was dissolved in *tert*-butyl methyl ether (1 ml) and vinyl acetate (103 mg, 0.11 ml, 1.2 mmol). This mixture was prepared in duplicate. Lipase SAM II was added to one vial and both were stirred at room temperature. Reaction was followed by monitoring by TLC, eluting with toluene: acetone 15:1. A spot on TLC corresponding to the product oxime acetate was observed in both the reaction and the control.

7.6 EXPERIMENTAL TO CHAPTER 6.

7.6.1 Preparation of the non-chiral enol acetate of 2-methylcyclohexanone

2-Methylcyclohexanone (10 g, 0.089 mole) was added to a mixture of *p*-toluene sulphonic acid (0.1 g) and acetic anhydride (18.3 g, 0.179 mole) and heated under reflux for 3 h. The resultant black oil was poured in 5% aqueous sodium bicarbonate solution (100 ml) and the product was extracted with ether (3 x 50 ml). The combined organic extracts were washed thoroughly with more sodium bicarbonate solution followed by water, dried over magnesium sulphate and evaporated. The remaining black oil was distilled at water pump pressure in a kugelrohr apparatus to give a single fraction, boiling at 120°C.

This was further purified by flash chromatography, eluting with ether: petrol 1:10.

Yield 47.5%.

^1H NMR (400MHz, CDCl_3): δ_{H} 2.09 (3 H, s, OAc), 2.05 (2 H, m, C(6)H) 2.00 (2 H, m, C(3)H), 1.67 (2 H, m, C(4)H or C(5)H), 1.58 (2 H, m, C(4)H or C(5)H), 1.48 (3 H, m, C(7)H).

^{13}C NMR (CDCl_3): δ_{C} 168.80 (C=O), 141.76 (C-1), 120.03 (C-2), 29.89, 26.95, 22.99, 22.32 (C-3, C-4, C-5, C-6) 20.55, 15.81 (CH_3)

Mass spectrum (+EI): m/z 154 (M^+), 112(100.0), 97(80.5), 84(51.9), 69(20.5), 55(12.2).

IR (liquid film): ν 2920, 2860s, 1750s, 1705w, 1220-1240s, 1105s cm^{-1} .

This compound has been fully characterised.³²⁰

7.6.2 Preparation of the chiral enol acetate of 2-methylcyclohexanone.

Diisopropylamine (6.77 g, 67 mmol) was dissolved in THF (50 ml, previously dried over Na/K) at 0°C under nitrogen. Butyllithium (1.6 M solution in hexanes, 42 ml, 67 mmol) was added and the mixture stirred at 0°C for 2 h. Tetramethylethylenediamine (7.85 g, 67 mmol) was added, the solution was stirred for 30 min and then cooled to -78°C. The 2-methylcyclohexanone (5 g, 45 mmol) was added and the solution stirred at -78°C. The reaction was followed by GLC by removing small samples, quenching with acetic anhydride and extracting with ether.

The ketone was seen to disappear after 90 min. Acetic anhydride (7 ml, excess) was added to the reaction mixture and stirring was continued at -78°C for 30 min and then at room temperature for 60 min. Water was added and the product extracted into ether (3 x 60 ml). The combined organic extracts were washed with 1M aqueous hydrochloric acid, dried over magnesium sulphate and evaporated. The product was purified by Kugelrohr distillation at water pump pressure. This yielded one fraction at 100-120°C. This material was further purified by flash chromatography, eluting with ether: petrol 1:10 and the product was obtained as a clear oil.

Yield 71%.

¹H NMR (400MHz, CDCl₃): δ_H 5.29 (1 H, 6 lines *J* 1.5, 4 Hz, C(6)H), 2.42 (1 H, m, C(2)H), 2.09 (3 H, s, CH₃CO), 2.06 (2 H, m, C(5)H), 1.84 (1 H, m), 1.60 (2 H, m), 1.38 (1 H, m) (C(3)H and C(4)H), 0.96 (3 H, d, *J* 7 Hz, C(7)H).

^{13}C NMR (CDCl_3): δ_{C} 169.27 (C=O), 151.74 (C-1), 114.01 (C-6), 31.27, 31.17, 24.02, 20.73 (C-2, C-3, C-4, C-5), 19.80, 17.92 (CH_3CO , C-7)

Mass spectrum (+EI): m/z 154 (M^+), 112 (100.0), 97 (79.7), 84 (62.3), 70 (56.7), 55 (31.1).

IR (liquid film): ν 2920, 2850s, 1750s, 1680m, 1450m, 1365s, 1220-1240s, 1100-1125s cm^{-1} .

This compound has been fully characterised.³²⁰

7.6.3 Preparation of the enol acetate from 2,6-dimethylcyclohexanone.

2,6-Dimethylcyclohexanone (10 g, 79.2 mmol) was dissolved in acetic anhydride (120 ml) and *p*-toluenesulphonic acid (0.4 g) was added. The mixture was heated under reflux for 24 h after which time GLC analysis of a small sample (quenched and extracted as in work-up procedure) showed that most of the ketone had disappeared. The reaction mixture was poured into cold 10% aqueous sodium hydrogen carbonate solution (200 ml). The product was extracted into petrol (3 x 100 ml) and the combined extracts were washed with further sodium hydrogen carbonate solution. The organic solution was treated with activated charcoal at room temperature to remove the dark colouration which had formed during reflux, dried over sodium sulphate and evaporated. The product was purified by flash chromatography, eluting with ether: petrol 1:15 and was obtained as a colourless oil.

Yield 55%.

^1H NMR (400MHz, CDCl_3): δ_{H} 2.38 (1 H, m, C(6)H), 2.12 (3 H, s, CH_3CO) 2.01 (2 H, complex t), 1.82 (1 H, m), 1.60 (2 H, m), 1.38 (1 H, m) (C(3,)H C(4)H, C(5)H), 1.47 (3 H, complex s, C(2)Me), 0.94 (d, 3 H, d, J 7 Hz, C(6)Me)
 ^{13}C NMR (CDCl_3): 168.87 (C=O), 145.41 (C-1), 120.30 (C-2), 31.61, 31.41, 30.50, 20.08 (C-3-C-6) 20.47, 20.08, 16.20 (CH_3).
Mass spectrum (+EI): m/z 168(M^+), 126 (100.0), 111 (89.4), 98 (0.8), 93 (23.4), 84 (72.2), 69 (34.9).
IR (liquid film): 1760s, 1705w cm^{-1} .
This compound has been fully characterised.³²³

7.6.4 Preparation of the enol acetate from norcamphor.

Diisopropylamine (13.66 g, 0.135 mole) was dissolved in THF (150 ml, previously dried over Na/K) at 0°C under nitrogen. Butyllithium (2.15M in hexane 62.79 ml, 0.135 mole) was added and the mixture stirred at 0°C for 2 h. Tetramethylethylenediamine (15.69 g, 0.135 mole) was added, the solution was stirred at 0°C for 30 min and then cooled to -78°C . Norcamphor (10 g, 0.09 mole) was added dissolved in more THF (10 ml). The solution was stirred at -78°C for 3 h. Acetic anhydride (15 ml, excess) was added and stirring was continued for 15 min. The mixture was warmed to room temperature and stirred for 1 h. Water (100 ml) was added and the product was extracted into ether (3 x 100 ml). The combined extracts were washed with 1M aqueous hydrochloric acid (2 x 100 ml) dried over magnesium sulphate and evaporated.

The resultant red oil was purified by flash chromatography, eluting with ether: petrol 1:1 to give the final product as a colourless oil.

Yield 51%.

^1H NMR (400MHz, CDCl_3): δ_{H} 5.47 (1 H, d, J 3 Hz, C(3)H), 2.86 (1 H, m), 2.81 (1 H, m), (C(1)H and C(4)H), 2.11 (s, 3 H, s, CH_3)

1.05-1.75 (6 H, m, CH_2 ring protons) minor impurities.

^{13}C NMR (CDCl_3): δ_{C} 168.41 (C=O), 156.42 (C-2), 115.03 (C-3), 47.07, 43.46, 40.90, 26.39, 24.42 (C-1, C-4, C-5, C-6, C-7), 20.89 (CH_3).

Mass spectrum (+EI): m/z 152 (M^+), 124 (7.4), 110 (8.1), 95 (7.0), 82 (100.0), 68 (7.0), 53 (18.2), 43 (81.1).

IR (liquid film): 2980, 2880s, 1770s, 1680, 1640 m, 1380, 1335 m, 1200-1250 s cm^{-1} .

Elemental analysis: Found C, 70.74; H, 8.19%. $\text{C}_9\text{H}_{12}\text{O}_2$ requires C, 71.03; H, 7.95%.

7.6.5 General Procedure for enzymatic hydrolysis of enol acetates from 2-methylcyclohexanone in a biphasic system.

The enol acetate (500 mg) was dissolved in isooctane (2.0 ml) and the appropriate lipase (200-300 mg) was dissolved in 0.06M phosphate buffer (4 ml). The two phases were stirred together and the extent of conversion was determined by the relative peak areas of the enol acetate and ketone on GLC.

The reaction was stopped once the desired conversion was achieved by separation of the phases. The aqueous phase was

extracted with ether and the combined organic phases were dried over magnesium sulphate and evaporated. The product and unreacted starting material were separated by Kugelrohr distillation at water pump pressure.

Result for hydrolysis of the chiral enol acetate with lipase from *Candida cylindracea*.

Ketone, yield 36.1 mg.

Analysis by GLC showed that the sample contained a trace of enol acetate.

$[\alpha]_D + 5.4^\circ$ (c 1.81, CHCl_3).

Result for hydrolysis of the chiral enol acetate with lipase from *Aspergillus niger*.

Ketone, yield 91.1 mg.

Analysis by GLC showed that the sample contained approx 20% enol acetate.

$[\alpha]_D + 4.4^\circ$ (c 1.4, CHCl_3).

No data is available for the rotation of the pure enantiomers of 2-methylcyclohexanone in chloroform. Previous measurements were made in MeOH but in this instance the samples did not fully dissolve in this solvent.

Hydrolysis of the non-chiral enol acetate in this solvent system with lipase from *Candida cylindracea* gave a crude isooctane phase with no apparent optical activity.

7.6.6 General procedure for the enzymatic transesterification of the enol acetate of norcamphor with n-pentanol.

The lipase (50 mg) was added to a solution of the enol acetate (140 mg) and pentanol (0.5 ml) in isooctane (4.5 ml). The resultant solution was stirred at room temperature and the extent of conversion determined from relative peak areas upon GLC analysis.

Once the desired extent of conversion was achieved the reaction was stopped by filtering out the enzyme using a glass sinter. The solvent was evaporated briefly using a cold water bath.

The enol acetate was separated by flash chromatography, eluting with ether: petrol 1:1.

The enol acetate (52 mg) was then dissolved in warm methanol (2.5 ml) containing concentrated aqueous hydrochloric acid (0.3 ml) and this mixture was heated for 10 min on a steam bath.

2,4-Dinitrophenylhydrazine (50 mg) was added and the solution heated for a further 10 min. After cooling, the DNP-norcamphor was filtered off, washed with ethanol and dried in a desiccator.

Result for the product from transesterification using lipase P.

DNP-norcamphor.

Yield 44 mg.

melting point 129-130°C (lit. 130-131°C).

optical rotation $[\alpha]_D +5.33$ (c 1 CHCl_3) ($[\alpha]_D$ of the pure enantiomer $+51^\circ$)

^1H NMR data was as previously reported for this compound.

Result for the product of transesterification using the lipase from
Candida cylindracea.

DNP-norcamphor.

Yield 30.5 mg.

Melting point 125.9-127.8°C (lit 130-131°C).

Optical rotation $[\alpha]_D$ 4.4° (c 1.42, CHCl₃).

CHAPTER 8.

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